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MOLECULAR CHARACTERISTICS OF EMERGING COLISITN RESISTANT GRAM-NEGATIVE BACILLI IN LEBANON

by
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AN ABSTRACT OF THE THESIS OF

Dana Mohamad Itani for Master of Science
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Title**:** Molecular Characteristics of Emerging Colistin Resistant Gram-negative Bacilli in Lebanon

Background: Gram-negative bacilli such as *Acinetobacter baumannii* and *Escherichia coli* are major causes of nosocomial infections worldwide. The misuse of broad-spectrum antibiotics contributed to the emergence of multidrug-resistant Gram-negative organisms (MDRGNs) that are of major threat to immunocompromised patients. The emergence of bacterial resistance against most classes of antibiotics and the shortage of antimicrobials against Gram-negative bacteria have led clinicians to reuse colistin, a polymyxin. Colistin targets the outer membrane of Gram-negative bacteria specifically anionic Lipopolysaccharide (LPS), a major component of their outer membrane. This leads to the disruption of the cell membrane and the displacement of magnesium (Mg^{+2}) and calcium (Ca^{+2}) ions leading to the leakage of the cell and consequently death. However, colistin-resistant isolates are increasingly reported with the usage of colistin, the last resort of antimicrobials. The recent emergence of colistin resistant isolates at the American University of Beirut Medical center led to the initiation of this study in order to assess the underlying mechanisms of resistance in colistin resistant Gram-negative bacilli.

Methods: Thirty-three non-selected MDR *Acinetobacter baumannii* and 5 Carbapenem resistant *Escherichia coli* clinical isolates from the Department of Pathology and Laboratory Medicine, American University of Beirut Medical Center (AUBMC), that included 7 MDR *A. baumannii* from Makased Hospital, were collected and used in this study. In addition, one *E.coli* and one *Enterobacter cloacae* isolates obtained from poultry were used. The levels of resistance were measured using disc diffusion technique, broth microdilution assay, E-test and Vitek system. Capsule staining for *A. baumanni* was done. Differences in the three-dimensional topography for colistin sensitive and colistin-treated resistant *A.baumannii* and *E.coli* were visualized by Scanning Electron Microscope. The genomic relatedness of these bacteria was determined by Random Amplified Polymorphic DNA (RAPD). Polymerase Chain Reaction (PCR) analysis was performed to detect colistin resistance encoding genes. These included, the lipid biosynthesis genes (*lpxA,lpxC,lpxD*), lipid A modifying genes (*pmrA,pmrB,pmrC*), porin (*$OmpA_{Ab}$ OmpW* genes, *in A. baumannii and OmpC OmpF* in *E.coli*), and *mcr-1 and mcr-2* genes in *E.coli*, in order to determine the mechanism of resistance. The level of resistance was further evaluated by measuring MIC

to colistin and detection by PCR of *mcr*-1 encoding gene, in a competent recipient *E.coli*, post transformation due to acquisition of MCR-1 plasmid.

Results: Thirty-one (94%) isolates of *A. baumannii*, 4 *E.coli and 2 E. cloacae* isolates exhibited resistance to colistin by BMD (MIC ≥ 4 ug/ml). In addition, 45.5% (15), 30% (10) and 24% (8) of our *A.baumannii* isolates were colistin resistanct by DD, E-test and Vitek respectively. Concerning *E.coli* and *E.cloacae* two isolates were resistant by DD and only one exhibited resistance by Vitek and E-test. Our data revealed 24% agreement between colistin resistant *A. baumannii* by DD, BMD, E-test, and Vitek. Minor concordance (45%) between DD and BMD were noted. However, major discrepancies were seen between Vitek, E-test and BMD especially at low MIC values. Capsule staining for *A. baumanni* deficient of LPS revealed an extensive halo of exopolysaccharide. As for the Scanning Electron Microscopy, isolates subjected to colisitn treatment showed perturbations, variation in size and in the septation of the cells. RAPD analysis showed genomic diversity among *baumannii* and *E.coli.* PCR analysis revealed that all *A. baumannii* isolates possess porin genes (*$OmpA_{Ab}$ OmpW*). On the contrary, three of *E.coli* isolates were shown to harbor the porin genes (*OmpC and OmpF*) and only one *E.cloacae* was shown to harbor *OmpC* porin gene. As for the Lipid biosynthesis genes, 11(34%), 32 (97%) and 10 (31%) of *A. baumannii* harbored *lpxA, lpxC and lpxD* respectively, while, all of *E.coli* clinical isolates harbored *lpxA and lpxC.* And 4 isolates were shown to harbor the *lpxD* gene. Similarly, for Lipid A modifying genes, 22 (67%), 31 (94%), 33 (100%) and 24 (72%) of *A. baumannii* isolates were shown to harbor *pmrA, pmrB, pmrC* and *eptA* respectively while all of *E.coli* clinical isolates harbored *pmrA, pmrB and pmrC* genes and none of *E.cloacae* were shown to harbor the Lipid A modifying genes .Double the percentage of *lpxD, lpxC*, and *pmrA, pmrB* and *pmrC* was noted between colistin sensitive and colistin resistant *A. baumannii.* In addition, all *A. baumannii* isolates were shown to have *pmrB* mutations except for one isolate that did not exhibit *pmrB T521A* mutation. Furthermore, none of the *A. baumannii* isolates harbored *pmrA* mutations and only one isolate was shown to confer resistance by insertion sequence *ISAba11.* As for the plasmid mediated colistin resistance, none of the *A. baumannii* isolates harbored *mcr-1*, while one *E. coli* isolate from poultry harbored this gene. Upon transformation of MCR-1 plasmid to colistin recipient competent sensitive *E. coli,* 16-fold increase in MIC equivalent to the parent cell was observed and the *mcr-1* gene was detected by PCR.

Conclusion: The alarming increase in colistin resistance over the past years, and the limitations in effective therapeutic approaches require a better understanding of resistance mechanisms. The different susceptibility testing methods yielded a wide range of discrepancies in resistance results. The 94% colistin resistant rate by BMD was not matched by the other tests. However, E-test and Vitek yielded very close resistant results but still were around 20% different from BMD. The encountered basis of resistance among the tested isolates indicated the involvement of several genes affecting the bacterial membrane remodeling. Molecular characterization of Gram-negative isolates demonstrated that such resistance is mediated by LPS loss and/or modifications primarily in *A. baumannii and Enetrobacter cloacae.* In this study, resistance in one *E.coli* isolate was due to MCR-1 plasmid harboring the *mcr-1* gene. Further investigations should be done in

order to tackle other factors that contribute to colistin resistance to get a better understanding of this life-threatening phenomenon in order to implement a suitable and effective combination therapy against LPS-deficient or LPS-modified cells.

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CHAPTER I

INTRODUCTION

The rise of infections caused by multi-drug resistant Gram-negative bacteria, such as *Acinetobacter baumannii* and *Escherichia coli*, represents an alarming threat to healthcare and patient safety worldwide. In the past decades, the world has monitored a rapid increase in resistance to penicillin and cephalosporins due to the emergence of Extended-spectrum Beta-Lactamase (ESBL). This has limited the therapeutic options to carbapenems. However, carbapenem resistance nowadays is on the rise worldwide. As a result, treatment options were narrowed to colistin.

Colistin was used 20 years ago against Gram-negative infections. It primarily acts by disrupting the bacterial cell membrane. However, it was abandoned due its nephrotoxicity and neurotoxicity. The increasing number of hospital outbreaks, the emergence of resistance to mainly all classes of antimicrobials, and the shortage of new antimicrobials have led the physicians to reuse colistin as the last resort for treatment purposes. The reintroduction of colistin to treat these infections was followed by high incidence of colistin resistance. These are mainly due to a number of mechanisms: chromosomal, adaptive and acquired. Chromosomal resistance is due to target modification and target loss. Target modification in Lipopolysaccaride (LPS) can be caused by mutations in the Two-component system; *pmrAB* locus resulting in the upregulation of *pmrC.* On the other hand, loss of Lipopolysaccaride (LPS) can be due to mutations ranging from deletions in first genes involved in Lipid A biosynthesis pathway *lpxA, lpxC, lpxD*, to insertion

sequence *ISAba11*. Adaptive resistance is due to the decrease or the loss in bacterial outer membrane proteins (OMP), permeability, and efflux pump expulsion of colistin. Acquired resistance is mediated by MCR-1 or MCR-2 plasmid transfer of the mobile colistin resistance genes from animal reservoir to humans.

Colistin resistant isolates have alarmingly increased worldwide. The ability of these isolates to survive the exposure to high concentrations of colistin and the epidemic dissemination of a plasmid-mediated genes has led The Centers for Disease Control and Prevention (CDC) and the European Antimicrobial Resistance Surveillance Network (EARS-Net) to take global actions to tackle this life-threatening problem and to prevent further spread of resistance.

The increase in colistin resistance at the American University of Beirut Medical Center (AUBMC) since 2012 contributed to the initiation of this project aiming at identifying the underlying molecular mechanisms of resistance of these isolates by a) visualizing by electron microscopy the morphological changes in both colistin resistant and susceptible *A. baumannii*, *mcr-1* positive isolate and susceptible *E.coli*, b) determining the genomic relatedness among colistin-resistant isolates using Random Amplified Polymorphic DNA (RAPD), c) assessing the mechanisms of resistance in clinical isolates by detecting LPS biosynthesis/modifying genes, mutation/insertion sequences, outer membrane porins and *mcr-1 mcr-2* plasmid mediated colistin-resistant genes, and d) demonstrating transfer of resistance in *E.coli* upon transformation of MCR1 plasmid into a recipient susceptible isolate

CHAPTER II

LITERATURE REVIEW

A. General Characteristics for *Acinetobacter baumannii*

Acinetobacter spp. are free-living saprophytes which are present in water and soil. Some of these species are common commensals of throat, skin and secretions of healthy individuals. *Acinetobacter* spp. are non-motile, strictly aerobic Gram-negative coccobacilli, that grow readily on solid media with optimum temperature of 33 – 37°C [1].

In the year 1970, *Acinetobacter* spp. was recognized as one of the significant healthcare-associated pathogens. Nowadays, *Acinetobacter* infections involve multidrug-resistant (MDR) strains, and extensively occur in the Intensive Care Unit (ICU) [2].

1. Complications

A. baumannii is the most frequently isolated species from human samples [3]. *A. baumannii* infections occur in different ranges and on different anatomical sites with varying severity and patient outcome. The overall mortality figures for *Acinetobacter* were the highest in the ICU [1]. The use of antibiotics, extended periods of hospitalization and prolonged exposure to mechanical ventilation increase the risk of Ventilator Associated Pneumonia (VAP). Contaminated ventilators as well as intra-hospital transmission due to poor personal hygiene of health care professionals may contribute to a nosocomial outbreak. Pneumonia due to an *Acinetobacter* infection can also occur outside the hospital setting due to a throat carriage [1].

In addition, *Acinetobacter* is a mostly reported as a pathogen of burns [4]. It is also becoming significantly involved in nosocomial, post-neurosurgical meningitis in comparison to other Gram-negative bacteria [1].

2. Treatment for *Acinetobacter baumannii*

Treatment of *Acinetobacter* infections is difficult since clinically important species are frequently resistant to commonly used agents, including aminoglycosides, broad-spectrum β-lactams and quinolones [2].

a. Carbapenems

Carbapenems are a class of broad-spectrum antimicrobials active against Gram-positive and Gram-negative bacteria, and anaerobes [5]. They act by inhibiting the cell wall synthesis. They remain one of the most important therapeutic choices for serious Gram-negative infections especially by ESBL producing isolates. They also have bactericidal activity and stability toward a range of β-lactamases. Recently, resistance to carbapenems as well as other antimicrobials is widespread, leaving colistin and tigecycline as the last therapeutic treatment options [6,7].

b. Tigecycline

Tigecycline is the first class of glycylcyclines. It has a bacteriostatic activity against multidrug-resistant *Acinetobacter* species [7,8]. It is a semisynthetic derivative of minocycline and inhibits the 30S ribosomal subunit. Its activity is not affected by

tetracycline-specific efflux pumps or tetracycline resistance determinants that protect the ribosome [9].

B. General Characteristics for *Escherichia coli*

The facultative anaerobic, Gram-negative rod-shaped bacteria *Escherichia coli* belongs to the large bacterial family, *Enterobacteriaceae*. *E. coli*, commonly found in the lower part of the intestine, is lactose fermenting and can grow both aerobically and anaerobically, preferably at 37ºC [10]. *E. coli* can be typed according to its lipopolysaccharide (O), capsular (K), and flagellar (H) antigens [11].

E. coli used to be considered as a harmless commensal [12]. Today, it is known to be responsible for major health problems worldwide. Several strains cause infections in the gastrointestinal system (Intestinal Pathogenic *E. coli*) while other strains can cause infections outside the gastrointestinal system (Extraintestinal Pathogenic *E. coli*) [13].

1. *Complications*

Most *E. coli* strains are harmless and they live in the normal flora. Pathogenic strains of *E. coli* cause a wide range of diseases mainly enteric diseases such as gastroenteritis, community acquired infections such as urinary tract infections, nosocomial infections, and neonatal meningitis, in addition to septicemia, pneumonia, and peritonitis. [13].

2. Treatment

E.coli infections are treated with a wide range of antimicrobials comprising β-lactams that include carbapenems and cephalosporins, fluoroquinolines, sulfonamides and

aminoglycosides [15]. With the rise of antibiotic resistance, carbapenems remain the treatment of choice for multidrug resistant infections and are administered due to their broad spectrum activity [16]. However, *E.coli* possessing β-lactamases that hydrolyze a wide range of β-lactams significantly imipenem, ertapenem and meropenem have caused the physicians to re-evaluate old drugs such as such as polymyxins, aminoglycosides and fosfomycin [17].

C. Gram-negative Bacteria Outer Membrane

The bacterial membrane acts as a barrier that protects the organism from harsh compounds such as antimicrobials.

1. Lipopolysaccharide

Lipopolysaccharide (LPS) is essential for the viability of most Gram-negative bacteria. [18]. The presence of this unusual glycolipid in the outer leaflet of the outer membrane, provides Gram-negative bacteria with a strong permeability barrier [19]. This accounts for the generally higher resistance of Gram-negative as compared to most Gram-positive bacteria to chemicals such as antibiotics and detergents. LPS are tightly packed at the outer membrane leaflet due to a strong interaction that occurs by the linking action of Mg^{2+} and Ca^{2+} divalent cations, thus, reducing the negative charges and stabilizing the structure [20]. LPS, a potent activator of the innate immune response, comprises lipid A (also known as endotoxin) which represents the pathogen associated molecular pattern (PAMP) recognized by innate immune receptors and activates complex signaling pathways that lead to the production of pro-inflammatory cytokines [21].

LPS is an amphipathic glycoconjugate molecule composed of three different domains:

- Lipid A, a hydrophobic domain that is anchored to the membrane and constitutes the endotoxic portion of the structure.
- The core oligosaccharide, which is divided into the inner core and the outer core, and connects the lipid A to the O-antigen. The inner core is connected to lipid A and consists of monosaccharides such as 2-keto-3-deoxy-octanoate (Kdo) and L-glycero-D-mannoheptose. The outer core is made of common sugars such as hexoses and hexosamines.
- The O polysaccharide is a hydrophilic oligosaccharide of repeated dideoxyhexoses units. It represents inter and intra-species variations [20,21].

a. LPS Biosynthesis

LPS biosynthesis occurs in three following compartments: the cytoplasm, inner membrane and periplasm [21]. It starts with the formation of lipid A in the cytoplasm at the internal face of the inner membrane. The first step in lipid A synthesis is the fatty acylation of UDP-*N*-acetyglucosamine (UDP-GlcNAc) by LpxA to UDP-3-*O*-acyl-GlcNAc which is deacetylated by the LpxC enzyme [20,21]. Furthermore, LpxD carries a second acylation reaction through a fatty acyl donor. Next, LpxH, LpxB and LpxK catalyze the formation of tetra-acylated lipid IV_A. Finally, the acyltransferases LpxL and LpxM catalyze the formation of Kdo_2-lipid A moiety. Additional sugar moieties are added to generate the oligosaccharide core. The core-Lipid A is flipped over to the periplasm by the ABC transporters. The formation of the mature LPS occurs after the addition of O-antigen

previously formed in the cytoplasm. Following synthesis, the newly formed mature LPS is transported to the outer membrane by the Lipopolysaccharide transport complex [22].

b. Lipid Biosynthesis genes

The LpxA, LpxC, LpxD proteins are coded in constitutive genes [20]. LpxB is a co-transcript with LpxA and catalyzes the formation of lipid A. LpxC is the committed step in lipid A biosynthesis [23].

c. Two-Component System Involved in the Alteration of Lipid A

The operon pmrCAB of *Salmonella,* similarly to that of *Acinetobacter,* is controlled by the Two-component system pmrAB [24,25]. Several studies suggest that there are over 100 genes regulated by PmrAB [21]. The Two-component system PmrAB in response to environmental pH, Fe^{3+} and Mg^{2+} levels, is activated where pmrB (kinase sensor), found on the cell membrane, becomes autophophorylated which in turns phosphorylates pmrA (response regulator) in the cytoplasm. pmrA, also acts a transcription factor, encodes proteins that include phosphoethanolamine phosphotransferase (PmrC). PmrC adds phosphoethanolamine to lipid A, a constituent of LPS, which imparts a positive charge on the outer membrane and affects the binding of antimicrobials (polymyxin) to its target [25,26]. In *E.coli,* PmrA in addition to pmrC activates other genes involved in the addition of 4-deoxy-aminoarabinose (Ara4N) in the core of the LPS. *A. baumannii* lacks the Ara4N biosynthesis and attachment genes [26].

2. Porins and Efflux pumps

a. Acinetobacter baumannii

Gram-negative bacteria display porins in their outer membrane that modulate the cellular permeability. Among of which is the Outer membrane protein A (OmpA). That is highly conserved among *Acinetobacter species* and is one of the most abundant β-barrel porins [27]. OmpA plays a role in the adaptation of *Acinetobacter* to the host niche. OmpA is a key player in the dissemination of *A. baumannii*, it facilitates its adherence and invasion to epithelial cells during infection [28]. Another porin found on the outer membrane of *Acinetobacter baumnanii* is OmpW, it allows the transport of Fe^{3+} to the cell [29]. Several studies have shown OmpW as an important player in colistin's mode of action where the bactericidal Dab residues of colistin bind to it and facilitates its action [30].

Efflux pumps are among the major mechanisms of resistance in *A. baumannii.* The antimicrobials are pumped outside the cell, which decreases the availability of the drug in the cell, thus decreasing its susceptibility [31,32]. Four efflux pumps are thought to be attributed to *A. baumannii* antimicrobial resistance these are the Resistance Nodulation Division (RND) family, Multidrug and Toxic Compound Extrusion (MATE) family, Major Facilitator Superfamily (MFS) and Small Multidrug Resistance (SMR) family of transporters [31].

a. E .coli

E.coli produces three major porins: OmpF, OmpC, and PhoE [33]. The loss of OmpC and OmpF has been linked to antimicrobial resistance in *E. coli.* Mutations in these two porin genes decrease the permeability of the membrane, lower the uptake of the drug and alter the

bacterial susceptibility [33]. Porins in general are non-specific, however, OmpF and OmpC prefer cationic substrates. In addition, OmpF has a larger porin channel in comparison to OmpC [34].

Efflux pumps, not only lowers the drug concentration by expelling it outside the cell, but also predispose the bacteria to tight regulations in response to environmental stimuli [35]. The major efflux pump is the RND superfamily [36].

D. Polymyxin

Polymyxin is a nonribosomal cyclic lipopeptide antimicrobial agent originally discovered in 1947. It is synthesized by *Bacillus polymyxa* subspecies *colistinus* Koyama [24]. The polymyxins include colistin (polymyxin E) and polymyxin B. Colistin was therapeutically used in the form of colistinmethanosulfate, but due to its nephrotoxicity and neurotoxicity, its use had greatly declined by the 1980s [37]. However, with the increasing reports of resistance worldwide, the emergence of bacteria resistant to all classes of antibiotics, and the shortage of new antimicrobials have led the physicians worldwide to reuse the old drug colistin.

1. Mode of action

The two drugs used in clinical practices are polymyxin B and polymyxin E (colistin). Polymyxins E_1 and polymyxins E_2 are the two major components of polymyxin E, and the amount of each depends on the pharmaceutical preparation [38]. As for polymyxin B it is mainly composed of polymyxin B_1 and polymyxin B_2. Another difference is that colistin is given by parenteral administration while in polymyxin B, the sodium salt in

colistinmethanosulfate needs to be hydrolyzed in vivo to form the active form of colistin [39]. Both share an almost identical primary sequence with a difference at position 6 where D-phenylalanine in polymyxin B is replaced by D-leucine in polymyxin E. Despite the slight difference in their structure, they exert the same action [24].

a. Membrane Lysis Death Pathway

Colistin possesses a narrow antibacterial spectrum mainly against Gram-negative bacteria [40]. It acts on the Lipid A portion of LPS. Through membrane lysis, the positively charged Dab residues in polymyxin interacts with negatively charged phosphates on Lipid A. This electrostatic attraction results in the displacement of Ca^{2+} and Mg^{2+} between adjacent LPS molecules leading to the destabilization of the outer membrane cell lysis and cell death [24].

b. Vesicle-Vesicle Contact Pathway

Vesicle-Vesicle Contact Pathway is another suggested mode of action for polymyxin [24]. Polymyxin can bind to both outer and inner membrane phospholipid leaflets and promote the exchange of phospholipids between vesicles. This leads to the loss of phospholipid specificity in the membranes, osmotic imbalance, and consequently cell lysis.

c. Hydroxyl Radical Death Pathway

A new report showed the possibility of polymyxin to induce cell death through the accumulation of hydroxyl radical (OH) [40,41]. It has been hypothesized, that when polymyxin molecules enter into and across the outer membrane, they will trigger oxidative

stress in the cell leading to oxidative damage of DNA, lipids, and proteins [24].

2. Mechanisms of Colistin Resistance in Gram-negative Acinetobacter baumannii and E . coli

Gram-negative bacteria can develop resistance to colistin, through different number of mechanisms that can be intrinsic, adaptive and/or acquired.

a. Intrinsic Resistance

i. Lipid A modification through Mutations in the Two-Component system

The most common mechanism of colistin resistance is through LPS modification [43]. Bacteria are able to modify the initial target (LPS) and reduce the electrostatic attraction by shielding phosphates of lipid A with positively charged phosphoethanolamine (pEtN) and L-4-aminoarabinose (L-Ara4N) [24]. *A. baumannii* does not possess the L-Ara4N genetic machinery; however, through its *pmrCAB* operon, it mediates the addition of PEtN to its lipid A [43].

Extensive studies have demonstrated colistin resistance due to mutations in *pmrA* and/or *pmrB gene* [43,44]. For *PmrA*, most mutations occurred in the phosphate receiver domain, while in *pmrB,* it occurred in the histidine kinase domain [45]. *PmrB* gene seems to be the most common site for bacterial mutations. Missense or in dell mutations in *pmrB* have been shown to induce the constitutive expression of *pmrA*. *PmrA* is responsible for the autoregulation of the operon *pmrCAB*, as a result, it induces the expression of pmrC, phosphoethanlomine transferase, that adds PEtN lipid A domain of LPS [24]. Some strains may have a higher tendency of becoming resistant since they

possess an additional *pmrC*-like gene (*eptA*) [46]. Phosphoethanlamine transefrase, eptA, adds a phosphoethanolamine on the core domain of LPS.

It is possible for *pmr* mutant colistin resistant strains to revert to their susceptible phenotype through compensatory mutations elsewhere in the *pmr* locus that counteract the hyper-activation of the Two Component System caused by the first mutation [47].

ii. LPS Loss through Mutations in Lipid Biosynthesis genes

The biosynthesis of lipid A is catalyzed by *LpxA, LpxC,* and *LpxD* [20]. Recent genome sequences have shown close arrangement of *lpxA* and *lpxD* in *A. baumannii* and in *E. coli* [48]. The other genes involved in lipid A biosynthesis are located elsewhere on the *A. baumannii* genome. Moffat et al. demonstrated another mechanism of resistance in *A. baumannii* through LPS loss. Mutations such as nucleotide substitution, deletion and an insertional inactivation with the insertion of *ISAba11* element in the first three lipid A biosynthesis genes, *lpxA*, *lpxC,* and *lpxD* result in the complete loss of LPS [48].

b. Acquired Resistance (Plasmid mediated)

Prior to the discovery of MCR-1 plasmid, colistin resistance has always been thought to be encoded chromosomally. Until late 2015, a surveillance report in China described for the first time a novel mechanism of transferrable colistin resistance [49]. The Mobile Colistin Resistant (*mcr-1)* gene located on a conjugative plasmid in *Escherichia coli,* pHNSHP45 isolated from a swine *E. coli* in southern China, and two other plasmids extracted from *E. coli* samples of patients with diarrhea. The plasmid pHNSHP45 is about 64kb long and it's isolated from swine, pE15004 is around 33kb and pE15017 (65kb) isolated from human

isolates [50]. This report was significant, not only for describing a novel mechanism of colistin resistance, but also for the detection of colistin-resistant *E. coli* in retail meat, pigs at slaughterhouses and in infected humans [50,51]. The fact that *mcr-1* positive bacteria was more frequently observed in animals and food of animal origin led to the suggestion that this new resistance gene had spread from the veterinary to the human domain and this is mainly due to the misuse of colistin in animal feeds. *mcr-1 gene* encodes a phophoethanloamine tranferase which reduces the net negative charge of the LPS through PEtN modifications in lipid A [51].

Following the report of MCR-1 plasmid in China, a study in Belgium showed the presence of a novel plasmid mediated colistin resistance MCR-2 in porcine and bovine colistin resistant *E. coli.* The *mcr-2* gene is 1,617 base pair long, nine bases shorter than *mcr-1,* and shows 76.75% nucleotide identity to *mcr-1* [52].

Phylogenetic analysis showed that MCR-2 encoded protein is distinct from that of MCR-1 with *Moraxella catarrhalis* being its origin. The latter is intrinsically resistant to polymyxin. *mcr-2* gene encodes a phosphoethanlomine transferase that catalyzes the transfer of phosphoethanloamine to lipid A moiety of LPS [52].

3. Colistin Antimicrobial Susceptibility Testing

Detection of colistin resistance in clinical isolates is challenging due to the lack of a definite susceptibility testing and polymyxin's unusual physiochemical features. Little is known regarding polymyxins mode of action, pharmacokinetics, and pharmacodynamics [53,54]. The variability of the powder drug between manufacturers is a contributing factor

that limits the development of an accurate susceptibility testing method.

Acquired colistin resistant *A. baumannii* and *E. coli* were shown to occur as a result of pre-exposure to colistin. Detection of colisitn resistance in *A. baumannii* is quite challenging due to heteroresistance and their ability to revert to their susceptible profile upon storage at -70 ^{o}C [55]. Current susceptibility testing methods used for polymyxin are Disk diffusion, Etest, Agar dilution, VITEK® 2 and Broth Microdilution (BMD) [56]. The large amphipathic structure of colistin hinders its ability to diffuse in the agar thus giving false positive results. [57]. Therefore, in 2017 The Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) agreed to remove the disk diffusion breakpoints and confirmed that disk diffusion can not be used for susceptibility testing of colistin [57]. As for colistin gradient tests like Etest, they underestimated MIC values leading to false susceptible values [58]. Broth Microdilution (BMD) remains the most preferred susceptibility testing for detecting colistin resistance [59]. Despite the ability of positively charged colistin to adhere to plastic microtiter plates consequently lowering the availability of the drug at lower concentrations. On the other hand, studies showed good agreement between agar dilution (AD) and BMD. Even though AD method is not routinely used in laboratories, it allowed the detection of heteroresistance. However, no studies have tackled the actual drug concentrations, nor the storage and stability of the drug in the AD plates [60].

CHAPTER III

MATERIALS AND METHODS

A. Source of *Acinetobacter baumannii and E. coli* isolates

Twenty-six non duplicate MDR *Acinetobacter baumannii* isolates were collected between 2012 and 2015 and 4 *E. coli* and 1 *Enterobacter cloacae* isolates were collected in 2016 and 2017, from the Clinical Microbiology Laboratory at the American University of Beirut Medical Center (AUBMC). In addition, 7 isolates of *Acinetobacter baumanii* were obtained from Al Makased General Hospital and 1 *E. coli* and 1 *Enterobacter cloacae* isolates obtained from poultry. The isolates were stored in Brucella Broth with 10% glycerol at -80^{o}C.

B. Bacterial Identification

Isolates were grown overnight at 37^{o}C on MacConkey Agar plates (BBL, Becton,Dickinson and Company, Sparks, Maryland). The following day, API 20 NE kit (bioMérieux®) used to identify non-fastidious, non-enteric Gram-negative rods was performed for *Acinetobacter baumannii* identification and API 20 E kit (bioMérieux®) was used to identify enteric Gram-negative rods such as *E.coli.* The profile of the bacteria was obtained after 24-hour incubation at 37^{o}C.

C. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed following the CLSI guidelines. As for the Disc diffusion method, Colistin sulphate discs (Oxoid™) were placed on Mueller

Hinton II Agar (BBL) following full bacterial streaking previously adjusted in double distilled water at 0.5 McF. The inhibition zone diameter was measured and classified resistant, intermediate or susceptible according to the CLSI breakpoints for all agents except colistin. Similarly, E test Colistin (bioMérieux®) strips were placed on Mueller Hinton II Agar (BBL). The MIC values were determined the next day by interpreting the zone of inhibition and classified resistant or sensitive according to Table 6.

To further confirm resistance, Broth microdilution was done and the minimum inhibitory concentration (MIC) was determined as the highest dilution with no visible growth. 100µL of Mueller Hinton Cation-adjusted broth (BD BBL™) was added to each well in the 96 well plate. A series of 2 fold serial dilutions was performed from a highest concentration of 256µg/mL to the lowest drug concentration 0.25µg/mL. The last column had no antimicrobial agent added and served as a negative control. 10µL of 10^8 CFU/mL bacteria adjusted to 0.5 McFarland, using a turbidometer (Densimat®), was added to each well. The plate was incubated overnight at 37 ^oC and results were recorded the next day.

E.coli ATCC25922 was used as a negative quality control strain and *E. coli* ECOL 1 harboring the *mcr-1* gene encoded on the MCR-1 plasmid from poultry origin, served as a positive quality control strains.

D. Capsule Staining

In order to distinguish the capsule material from bacterial cell wall positive capsule stain was done using 20% Copper (II) sulfate and 1% Crystal violet.

1. Materials

- 1% Crystal Violet
- 20% Copper(II) sulfate (Merck)
- Bacterial samples
- Distilled water

2. Protocol

- A drop of distilled water was added on a glass slide and bacterial samples, previously grown on MacConkey plates, were spread using an inoculation loop.
- The samples were left to air-dry and were not heated to prevent any possible bacterial shrinkage.
- Crystal violet was added and left to stand for 5 minutes.

After which, the samples were rinsed with copper sulfate and visualized using a light microscope at 1000x magnification.

E. Scanning Electron Microscopy

For Scanning Electron Microscopy (SEM) analysis bacterial isolates adjusted at 1 McF were incubated overnight at 37°C. Colistin resistant *E.coli* and *Acinetobacter baumanii* were subjected to 4μg/mL Polymyxin. Samples were processed and imaged using the Scanning Electron Microsocopy X-Max Oxford instrument.

1. Materials

- 2.5% Glutaraldehyde (GA)
- 2% Formaldehyde (FA)
- Phosphate Buffered Saline (PBS): 5mM K_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.0)
- Formvar-carbon coated Electron Microscope (EM) grids

2. Protocol

- The overnight cultures were centrifuged at 6,000xg for 2 minutes and the supernatant was discarded.
- To each pellet 50µL 2% FA (Sigma-Aldrich Co, St Louis, Missouri) was added for primary fixation.
- 10µL of the samples were deposited on Formvar-carbon coated EM grids. The samples were incubated at room temperature for 25 minutes.
- The Carbon coated EM grids were washed with an equal volume of PBS.
- The grids were then transferred to 50µL of 2.5% Glutaraldehyde (GA) for secondary fixation and incubated for 25 minutes at room temperature.
- The grids were washed four times with equal volumes of PBS
- The samples were left to air dry to prevent any further shrinkage of bacterial samples.

- The fixed-dried samples were platinum coated 6nm in thickness 21.45g/m^3 at a current of 15mA for 5 minutes using the platinum Q150T Turbo-pumped Sputter Coater/Carbon Coater.
- The samples were visualized using InBeam SEM detector at 10.00 kV, magnification of 23.07 kx, and 5.99μm view field.

F. Total DNA Extraction

DNA was extracted from 33 *A. baumnnii* colistin resistant strains and 5 *E. coli* and 2 Enterobacter colistin resistant isolates using the Qiagen QIAamp DNA mini kit (50) and according to the protocol for purification of genomic DNA for Gram-negative bacteria.

1. Materials

- ATL Buffer (Total Lysis Buffer)
- Proteinase K
- Buffer AL (Lysis Buffer)
- Ethanol 96%
- Minispin column
- Collection tubes
- Buffer AW1 (Wash Buffer)
- Buffer AW2
- AE Buffer (Elution Buffer)

2. Protocol

a. Bacterial culture

From previously grown bacteria on MacConkey Agar plates, $3x10^8$ cells/µL were inoculated in Luria Bertani broth (BD BBL™) and incubated overnight at 37°C. 1mL of the bacterial suspension were transferred into a microcentrifuge tube. After which they were centrifuged at 5000g for 5 minutes. Later, the supernatant was discarded.

b. Lysis

- 170µL of ATL buffer were added to each bacterial pellet to obtain a total of volume of 180µL.
- 20µL of proteinase K were added, vortexed and incubated at 56°C for 30 minutes (interrupted every 15 minute by vortexing). The 1.5mL microcentrifuge tubes were centrifuged to remove drops from inside of the lid.
- 200µL of Buffer AL were added to each of the samples and the solutions were then vortexed for 15 seconds and incubated at 70°C for 10 minutes. The microcentrifuge tubes were centrifuged to remove drops from inside of the lid.

c. Purification

- 200µL of Ethanol (96%) were added to each tube followed by vortexing for 15 seconds. The latter were then centrifuged.

- Each sample were then transferred to a QIAamp Mini spin column without wetting the rim and centrifuged at 6000g for 1 minute. The filtrate was then discarded and the Mini column was placed into a new 2mL collection tube.

d. Wash and dry

- 500µL of Buffer AW2 were added to each mini column and centrifuged at 20000g for 3 minutes. Afterwards, the filtrate was discarded and the mini columns were placed into new collection tubes.
- To completely eliminate the AW2 Buffer, the samples were spun again at full speed 20000g for 1 minute.
- The mini columns were then placed in clean 1.5mL microcentrifuge tubes.

e. Elution

- 200µL of AE Buffer were added to each column and incubated at room temperature for 5 minutes.
- Then the samples were centrifuged at 6000g for 1 minute, after which, the columns were discarded and the microcentrifuge tubes were stored at -20°C for further DNA quantification.

3. DNA quantification

The concentration and purity of the DNA samples were measured using the Thermo Scientific NanoDrop™ 1000 spectrophotometer. After choosing the Nucleic Acid software, the samples were pipetted directly to the optical measurement surface.

G. Plasmid Extraction

The plasmid was extracted from an MCR-1 positive *E. coli* isolate using the GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich Co, St Louis, Missouri).

1. Materials

- Resuspension Buffer
- RNase A
- Lysis Buffer
- Neutralization buffer
- Binding Buffer
- Column preparation solution
- Wash solution (1)
- Wash solution (2) diluted with 95% ethanol
- Elution solution (10 mM Tris-HCl, 1 mM EDTA, pH 8)

2. Protocol

0.5 McF *E. coli* were incubated overnight at 37°C 250-300 rpm in LB broth containing 2μg/mL Polymyxin E.

a. Cell Lysis and Homogenization

- 1.9mL of the overnight culture were transferred into an autoclaved microcentrifuge tube and spinned at 12,000xg for 15 minutes.

- The supernatant was discarded and 200µL of Resuspension buffer with RNase A were added. The mixture was pipetted up and down and vortexed to obtain a homogenous mixture.
- 200µL of Lysis buffer were added and the microcentrifuge tube was gently inverted up and down (6-8 times) to prevent genomic shearing and contamination.

b. Neutralization

- The cell debris were precipitated by adding 350µL of Neutralization or Binding solution. the tubes were gently inverted (4-6 times).
- The Pellet (containing proteins, lipids, SDS gDNA) was sedimented by centrifugation at 12,000xg for 10 minutes until a clear supernatant was obtained.

c. Column Preparation

After the assembly of the column to the tube, 500µL of the Column Preparation Solution were added and centrifuged at 12,000xg for 1 minute. The flow-through was discarded.

d. Washing and Elution

- The clear lysate was then transferred to the previously prepared column and centrifuged at 12,000xg for 1 minute. The flow- through was discarded.
- 500µL Wash solution (1) were added to lower the levels of endotoxins. Centrifuged at 12,000x g for 1 minute. The flow-through was discarded.
- 750µL of Diluted Solution (2) were added and centrifuged at 12,000xg for 1 minute to

remove extra ethanol.

- The column was transferred to an autoclaved tube and 100µL of Elution Solution were directly added to the filter and centrifuged at 12,000xg for 1 minute. The plasmid is now found in the elute and immediately stored at -20°C.

3. ***Plasmid quantification and purity*** was determined by Thermo Scientific NanoDrop™ 1000 spectrophotometer with a ratio (A260/A280) between 1.7 to 1.9.

H. Random Amplified Polymorphic DNA (RAPD)

In order to determine the relatedness of the samples, Random Amplified Polymorphic DNA (RAPD) was carried out by which a random fragment of DNA was amplified using Illustra™ Ready-To-Go™ RAPD Analysis Beads and amplified using the Bio-Rad C1000 Thermal Cycler.

1. Materials

- Primer 2 (5'-d[GTTTCGCTCC]-3') reconstituted in 500µL distilled water and stored at -20°C.
- DNA template
- Distilled water
- Ready-To-Go RAPD Analysis Beads containing thermostable polymerases, dNTPs (0.4 mM each dNTP in a 25µL reaction volume), BSA (2.5 µg) and buffer [3 mM MgCl2, 30 mM KCl and 10 mM Tris, (pH 8.3)

- Lyophilized control *E. coli* C1a DNA suspended in 200µL distilled water to obtain a final concentration of 5pmol/Ml

2. Protocol

- 15µL of distilled water were added to the beads of the tube of RAPD Analysis Beads followed by 5µL of reconstituted Primer 2 and 5µL of template DNA to obtain a total volume of 25µL.
- The contents were then mixed by pipetting up and down and centrifuged to collect the contents at the bottom of the tube.
- Subsequently, the DNA was amplified according to the following cycling conditions:

1 cycle at 95°C for 5 minutes, followed by 45 cycles of: 95°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes.

I. Gel Electrophoresis

After amplification, the RAPD products were visualized on a 2% agarose gel.

1. Materials

- TBE (Tris Boric EDTA) from 10xTBE buffer (prepared as followed 108g Tris base acid, 55g of Boric acid, and 9.3g of disodium EDTA dissolved in 1L distilled water and then autoclaved).
- SeaKem® LE Agarose (Lonza)
- 0.625 mg/ml Ethidium bromide (Amresco, USA)
- 6x Loading dye (Fermentas, USA)

- 100bp DNA ladder (Fermentas, USA)

2. Protocol

- 2% agarose gel was prepared by adding 6g of Seakem LE agarose into 300mL 1xTBE
- The agarose mixture was then microwaved for 5.5 to 6 minutes with interrupted shaking.
- 21µL of 0.625mg/mL of Ethidium Bromide was added to the agarose gel.
- After the assembly of the electrophoresis machine, the hot gel was poured. Within 30-45 minutes the gel was solidified and submerged with 1xTBE and 10µL of 5mg/mL of Ethidium Bromide.
- The samples and the 100 base pair ladder were loaded into the wells (2µL dye with 10µL RAPD product and 7µL of 100bp ladder with 5µL 1xTBE).
- Finally, the gel was run at 150V for 3 hours.

Gel Doc™ EZ System was used to visualize and photograph the bands.

J. Polymerase Chain Reaction

Polymerase Chain Reaction was carried out to amplify and detect the presence of the *mcr*-1, *mcr*-2, lipid biosynthesis genes *lpxC, lpxD, lpxA,* operon genes *pmrA, pmrB and pmrC* genes, Mutations, insertion sequence *ISAba11,* porin genes *OmpC OmpF* $OmpA_{Ab}$ *OmpW* in *Acinetobacter baumanii* and *E.coli* (Table 1).

1. Materials

- 10x TopTaq DNA polymerase buffer
- 25µM Magnesium Chloride
- 210µM dNTPs

(500µL were prepared by adding 10µL of 100mM dATP, 10µL of 100mM dGTP, 10µL of 100mM dCTP, and 10µL of 100mM dTTP to 460µL nuclease free water)

- Nuclease Free water
- 5U/µL TopTaq DNA polymerase
- DNA extract
- Forward and Reverse Primers
- TE (Tris-EDTA)

Both primers were reconstituted in 50µL 1xTE buffer to obtain a final concentration of 100µM.

2. Protocol

The master mix was prepared so that each sample would contain a total volume of 47.5µL of which:

- 26.25µL nuclease free water
- 5µL 10x Taq polymerase buffer
- 5µL Forward Primer + 5µL Reverse Primer
- 4µL MgCl2
- 2µL dNTP

Finally, 2.5µL of DNA were added to each PCR tube to obtain a final volume of 50µL except for the negative control tube in which 2.5µLof nuclease free water were added. Bio-Rad C1000 Thermal Cycler was used.

K. Gel Electrophoresis

1. Materials

- SeaKem® LE Agarose (Lonza)
- TBE
- 10 mg/ml Ethidium bromide (Amresco, USA)
- 6x Loading dye (Fermentas, USA)
- 100 bp DNA ladder (Fermentas, USA)

2. Protocol

- 1.5% agarose gel was prepared by adding 1.5g of SeaKem® LE Agarose into 100mL 1xTBE (Tris Boric EDTA) from 10xTBE buffer.
- The agarose mixture was then microwaved for 1.5 minute with interrupted shaking.
- 2 drops of Ethidium Bromide were added to the microwaved agarose gel.
- After the assembly of the electrophoresis machine the hot gel was poured. Within 30-45 minutes the gel was solidified and submerged with the remaining 900mL 1xTBE.
- The samples and the ladder were loaded into the wells (2µL dye with 10µL PCR product and 7µL of ladder with 5µL 1xTBE).
- Finally, the gel was run at 120V for 1 hour 45 minutes.

Gel Doc™ EZ System was used to visualize and photograph the bands.

L. Transformation by Heat Shock

The MCR-1 plasmid was transferred to competent colistin susceptible *E. coli* using Heat Shock method.

1. Materials

- MCR-1 plasmid previously extracted
- Competent colistin susceptible *E. coli DH5-* α
- Heat block set at 42°C
- Sterile Luria Broth (BBL™)
- LB agar (BBL™) prepared with 4µg/mL of polymyxin E
- LB broth (BBL™) prepared with 4µg/mL of polymyxin E

2. Protocol

- 100µL of competent *E. coli* were thawed on ice and 100ng to 1µg of Plasmid was added to the bottom of the tube. The Mixture was gently flicked.
- The bacteria were set on ice for 30 minutes then in the Heat block at 42°C for 90 seconds.
- Followed by 2 minutes on ice
- The transformed bacteria were then transferred into LB broth without antibiotic and incubated for 37°C at 90-250 rpm for 2 hours.

- After which they were transferred into a beaker containing LB broth and polymyxin E and left in the shaker incubator overnight at 37°C.
- The next day part of the transformed bacteria was centrifuged at 36,000 rpm for 30 minutes, the supernatant was discarded and the obtained pellet was frozen at -20°C.
- 500µL of the overnight transformed bacteria were added to 500µL of 80% Glycerol and stored at -80°C.

The transformation experiment was verified by plasmid extraction, PCR for *mcr-1* gene and determination of the MIC for the parent, conjugant, and bacteria transformed on agar and in broth (refer to materials above).

CHAPTER IV

RESULTS

A. Antimicrobial Susceptibility

Thirty-three *Acinetobacter baumannii* isolates isolated from different sources were subjected to antimicrobial susceptibility testing. Ninety-four percent were colistin resistant by Broth Microdilution (BMD). On the other hand, 27% of which were resistant by E-test and 45% were resistant by disk diffusion DD. As for 5 *E. coli* isolates collected from 2016 to 2017, 85% of which were colistin resistant by BMD, 33% isolate were resistant by E-test and 6% by DD (Table 4,5,6).

These isolates were subjected to further screening tests.

Our data revealed 24% agreement between colistin resistant *A. baumannii* by DD, BMD, E-test, and Vitek. Minor concordance (45%) between DD and BMD were noted. However, major discrepancies were seen between Vitek, E-test and BMD especially at low MIC values (Table 7).

B. Capsule Staining

The capsule for LPS deficient multi-drug resistant *Acinetobacter baumannii* ACN1 was visualized under light microscopy revealed an extensive exopolysaccharide formation which was seen as a halo around the bacteria. In contrast to the ACN33 that displayed a thin capsule and bacterial cells were more clustered (Fig. 2).

C. Scanning Electron Microscope

Scanning electron microscope was done in order to examine the morphological changes of *A. baumannii* colistin and *E.coli* susceptible and resistant isolates. Four μg/mL of polymyxin was used to treat colistin resistant *Acinetobacter baumannii* ACN1 and MCR-1 positive *E.coli* ECOL1. ACN1 were more spherical in comparison to colistin sensitive *A. baumannii*. On the other hand, slight morphological changes such as perturbations were seen in MCR-1 positive ECOL1 in comparison to sensitive control strain ATCC 25992. Upon colistin treatment, differences in cellular morphology were visualized roughened and grooved, variations in the size of individual cells and in the septation of the cells (Fig. 3,4)

D. RAPD Analysis

In order to determine the genomic relatedness of the *A. baumanii* and *E.coli* isolates Random Amplified Polymorphic DNA (RAPD) analysis was done. RAPD showed genomic variability between isolates. Nine different patterns for *A. baumannii* isolates were demonstrated these were: ACN1,2,3,4,5 were shown to have genomic similarity, similarly for ACN19,20,24,29,32,26, ACN18,26, ACN13,15, ACN14,23, ACN26,27,30, ACN8,9,7, ACN10,11 and ACN16,17. However, isolates ACN12, ACN21, ACN25, ACN31 and AN33 are different strains.

Concerning *E.coli* and *E.cloacae* isolates, each had a different pattern. (Fig 5).

E. Detection of Porin encoding genes by PCR

1. A. baumannii

PCR done on total DNA extract for colisitn resistant and susceptible *Acinetobacter baumannii* revealed that all of the clinical isolates harbored the major porin gene found in *Acinetobacter* $OmpA_{ab}$ *or OMPHMP*. As for *OmpW,* PCR amplification followed by gel electrophoresis showed that 100% of *Acinetobacter baummnii* were positive for this gene (Fig. 6).

2. *E. coli*

Similarly, *E. coli* clinical isolates screened for *OmpC and OmpF* porin genes encoding porins for cationic antimicrobials such as Colisitn. PCR amplification of our clinical isolates showed variability in gene detection, 3 of our *E.coli* isolates *to* harbor *OmpF and OmpC,* respectively. And only one *E. cloacae* ECOL5 was shown to harbor *OmpC.*

F. Detection of LPS encoding genes

1. Lipid A Modifying genes

- *pmrB,* which encodes for a senor kinase, PCR amplification revealed that only 2 isolates ACN1 and ACN2 do not harbor this gene and 94% harbored this gene (Fig. 6)

On the other hand, all *E.coli* isolates were shown to harbor the *pmrB* gene and none of our *E. cloacae* were shown to harbor this gene: ECOL1,3,4,6 and ECOL7.

- *pmrA* which encodes for a response regulator and a Transcription factor, 67% of *Acinetobacter baumanni* harbor this sequence 33% didn't harbor this gene.

However, PCR amplification for *E.coli* and *E.cloaca*e revealed all of our *E.coli* isolates to harbor this gene of isolates to harbor this gene: ECOL1,3,4,6 and ECOL7.

- *pmrC/eptA, pmrC* encodes a phosphoethanloamine transferase which adds a phosphoethanloamine to Lipid A. Moreover, *eptA* encodes for a phosphoethanoamine transferase that adds phosphoethanloamine to core-Lipid A. All *A. baumannii* isolates possessed *pmrC* gene however varied in *eptA* presence 72% harbored this gene. Concerning *E. coli* and *E. cloacae,* all of our *E.coli* isolates were shown to harbor this gene these were: ECOL1,3,4,6 and ECOL7.

2. Lipid Biosynthesis genes

- *lpxA,* encodes Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase that catalyzes the fatty acylation of UDP-N acetylglucosamine, 34% of *Acinetobacter baumannii* isolates harbored *lpxA* and 66% did not harbor this gene (Fig. 6). As for *E.coli* and *E. cloacae* isolates PCR amplification revealed all of our *E.coli* isolates to harbor this gene: ECOL1,3,4,6 and ECOL7.
- *lpxC,* encodes for UDP-3-O-acyl-N-acetylglucosamine deacetylase which is the committed step of lipid A biosynthesis, only one *Acinetobacter baumanii* isolate ACN1 didn't harbor this gene and 97% of *A. baumannii* harbored *lpxC* gene. Regarding *E.coli* and *E. cloacae,* All of our *E. coli* isolates and one *E.cloacae* harbored this gene: ECOL1,2,3,4,6 and ECOL7.
- *lpxD,* encodes UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferase, 31% of *A. baumannii* harbored *lpxD* genes and 70% did not harbor this gene. Regarding *E.coli*

and *E. cloacae*, PCR amplification revealed ECOL3,4,6,7 and *E.cloacae* ECOL5 to harbor this gene.

G. Detection of Mutations in *pmrA*, *pmrB* and Insertion sequence *ISAba11* in LPS encoding genes

PCR amplification of nonsynonymous mutations *pmrA* A24*T*, confirming mutation in phosphate receiver domain of pmrA, showed no mutations in our isolates. As for *pmrB* mutations, *pmrB* G788T *pmrB* T346C mutations were found in all *Acinetobacter* isolates even in the colistin susceptible isolate. However, *pmrB T521A* mutation was found in all isolates except for ACN33 (Fig. 7).

Insertion sequence *ISAba11*, part of the transposon Tn6021, is both mobile and replicative and the movement of this mobile element results in increase levels of colistin resistance. Only one *Acinetobacter* ACN12 was found to carry this insertion sequence in their *lpx* genes and resulted in the total loss of LPS.

H. Distribution of Genes by Different Antimicrobial Susceptibility Testing

1. Disc Diffusion Technique

Out of the 15 colistin resistant *A. baumannii* by disk diffusion susceptibility testing, 5 (33%) of the isolates harbored LPS biosynthesis genes *lpxD*, 4 (26%) harbored *lpxA* and 14 (93%) harbored *lpxC*. As for the lipid A modifying genes, 9 (60%) of the isolates possessed *pmrA*, 13 (86%) possessed *pmrB*, all of our isolates possessed *pmrC* and 11 (73%) of *A. baumannii* isolates harbored *eptA*. Our data revealed double the percentage of LPS

biosynthesis genes (*lpxA* and *lpxC*) lipid A modifying genes (*pmrA, pmrB* and *pmrC*) in colistin sensitive isolates in comparison to colistin resistant isolates (Table 8).
Similarly for *E.coli* and *E.cloacae,* 2 isolates were shown to be resistant by DD of which non harborerd *lpxD* and both harbored lpxC. Only one isolate harbored *lpxA, lpxC, pmrA, pmrB, pmrC and mcr-1*.

2. E-test

Similar distribution of genes was noted by E-test, 30% of our *A. baumannii* isolates were colistin resistant by E-test. 10 (40%), 3 (30%) and 9 (90%) of our isolates were shown to harbor the LPS biosynthesis genes *lpxD, lpxA* and *lpxC,* respectively. As for the lipid modifying genes, 8 (40%) of the colistin resistant isolates were shown to harbor *pmrA*, 8 (80%) were shown to harbor *pmrB,* all of our colistin resistant isolates were shown to harbor *pmrC* and 7 (70%) of our isolates were shown to harbor *eptA.* Similarly to resistance by DD, double the percentage of LPS biosynthesis genes and lipid A modifying genes in colistin sensitive isolates in comparison to colistin resistant isolates was noted (Table 8).
On the other hand, only one isolate from *E.coli* and *E.cloacae* was shown to be resistant by E-test and this isolate only harbored *lpxC*.

3. Vitek

Twenty-four percent of our isolates were colistin resistant by Vitek, of these 4 (50%) harbored the *lpxD* gene, 3 (37%) harbored the *lpxA* and 7 (87%) harbored the *lpxC* LPS biosynthesis genes. On the other hand, 3 (37%) of colistin resistant isolates by Vitek harbored Lipid A modifying genes *pmrA*, 5 (62%) harbored *pmrB*, all of our isolates

harbored *pmrC* genes and 7 (87%) of harbored the *eptA* gene. Also, colistin sensitive isolates were shown to harbor double the percentage of LPS biosynthesis genes and lipid A modifying genes in comparison to that of colistin resistant isolates (Table. 8).

Furthermore, only one isolate was shown to be colistin resistant in *E.coli* and *E.cloacae* and this isolate only harbored *lpxC*.

4. Broth Microdiltuion Assay

Ninety-four percent of *A. baumannii* isolates were colistin resistant by BMD, 10 (32%) harbored each of *lpxA* and *lpxD* genes. Concerning the third LPS biosynthesis gene *lpxC*, 30 (97%) of the isolates were shown to harbor this gene. As for the Lipid A modifying genes, 20 (64%), 29 (93%), 33 (100%) and 23 (74%) were shown to harbor each of *pmrA*, *pmrB, pmrC* and *eptA,* respectively. Furthermore, colistin sensitive isolates were shown to harbor double the percentage of LPS biosynthesis genes and lipid A modifying genes in comparison to that of colistin resistant isolates (Table 8.).

Moreover, 6 isolates *E.coli* and *E.cloacae* isolates were colistin rsesitant by BMD. Four of which harbored *lpxD, lpxA, pmrA, pmrB, pmrC* and *mcr-1*.

I. Detection of *mcr-1 and mcr-2* genes

PCR amplification of *mcr-1* gene in *A. baumannii* and *E. coli* revealed no *A. baumannii* isolates to possess this gene. As for *E.coli* isolate, only one ECOL1 harbored this gene. In addition, *mcr-2* was not detected in neither *A. baumannii* nor *E.coli* (Fig. 7).

J. Transformation by Heat Shock

Transformation experiment showed upon the transfer of resistance via MCR-1 plasmid to competent *DH-5 alpha* colistin susceptible *E. coli.* The transformed *E.coli* was able to grow on LB agar containing 4µg/mL of Polymyxin E and in LB broth containing similar concentrations of Polymyxin E overnight in shaker incubator. The successful transformation was verified by MIC and PCR. The MIC value of the transferred colistin susceptible *E. coli* increased from 0.25µg/mL to 16µg/mL in bacteria grown in broth and on agar which was equivalent to the parent. MCR-1 was shown to increase colistin MIC 16-fold when transmitted to susceptible isolates as seen by Liu *et al* [31]. The plasmid extracted from these transformed *E. coli* was amplified by PCR for *mcr-1* gene and visualized on ethidium bromide stained gel (Fig. 8)

Table 1. PCR Primers for the detection of colistin resistant genes, Porins, and Insertion sequence

GENE	PRIMER SEQUENCE (5'-3')	AMPLICON SIZE	REF
MCR-1	F: CGGTCAGTCCGTTTGTTC R: CTTGGTCGGTCTGTAGGG	309bp	[49]
MCR-2	F: TGGTACAGCCCCTTTATT R: GCTTGAGATTGGGTTATGA	1617bp	[52]
$LPXA_{AB}$	F:ATTCAAGGATCCCACCTCGAGCATTGTACCA R:ACGCCAGGATCCGGTTCATTATTCCTGTTTGCT	1176bp	[48]
$LPXD_{AB}$	F: CAAAGTATGAATACAACTTTTGAG R: GTCAATGGCACATCTGCTAAT	1502bp	[48]
$LPXC_{AB}$	F: TGAAGARGACGTTCCTGCAA R: TGGTGAAAATCAGGCAATGA	137bp	This study
$PMRA_{AB}$	F: ATGACAAAAATCTTGATTGAAGAT R: TTATGATTGCCCCAAACGGTAG	675bp	[46]
PMRA A24T	F: GCCAACAAACACATATTC R: TTTGACGGATTTGCTTTA	413bp	[46]
$PMRB_{AB}$	F: CCTAAATCGARRRCTTTTTG R: GGTTCGTGAAGCTTTCG	505bp	[46]
PMRBG788T	F: ATTATTCGTCGTGGTTTA R: AGTAGGTTCAATACTATGC	503bp	[46]
PMRBT346C	F: TGAAGTATTTAGCGGAAA R: GCGTTCTTTTAACTCAT	412bp	[46]
PMRBT521A	F: GTTATATGACTTTGATAGTGATG R: GATCTTCTTCGTGGTAAG	374bp	[46]

PMRC$_{AB}$	F: GGTCGGTGTTTTACTTTTTACCTA R: CATCCCTTTTAAATCACGATGT	75bp	[46]
ISABA11	F: TAGGACTTACGCATTGAC R: TAGGACTTACGCACTATCATTTAT	1101 bp	[61]
OMP$_{AB}$	F: CTCTTGCTGGCTTAAACG R: TGTGTGACCTTCGATACG	254 bp	[62]
OMPW	F: TTAGCATCAGCAGGTTGG R: TATTGGTATCGGGGCAAC	120 bp	[62]
PMRA$_{E}$	F: GTGAGGATCAGTACCGGCAG R: AACAAAGCCTTGAGGCATGGT	127bp	This study
PMRB$_{E}$	F: AGGCTGACCATAAAGACGCC R: CCATCGGGGCCATTTTGTTG	187bp	This study
PMRC$_{E}$	F: TTTGCAGGCAGTTCTGGTCA R: TGGCATCTATCTGCACGGTC	125bp	This study
LPXC$_{E}$	F: CTATCGTCGCACCGACTTGA R: GCTCGTTGACCAGACACGTA	95bp	This study
LPXA$_{E}$	F: CATGACCGCAGTCCATCAGT R: GTAATCGCCTCACGGCTGAA	174bp	This study
LPXD$_{E}$	F: GCAGAACATTGCACCCAGTG R: AGTTCAACGCCGGACTCAAT	99bp	This study
OMPF	F: CAGGTACTGCAAACGCTGC R: GTCAACATAGGTGGACATG	953bp	[63]
OMPC	F: GAACTGGTAAACCAGACCCAG R: GTTAAAGTACTGTCCCTCCTG	1086bp	[63]

Table 2. Antimicrobial susceptibility profile of *Acinetobacter baumnnii* isolates TIG: Tigecycline, IMP: imipenem, CFP: Cefepime, CTZ: Ceftazdime,
S: susceptible, R: resistant, -:Unknown

					Antimicrobial agent			
Isolate #	**Code#**	**Sex/age**	**Origin**	**Year**	**TIG**	**IMP**	**CFP**	**CTZ**
2090	ACN1	M/65	Catheter	2015	R	R	R	R
2285	ACN2	M/74	Throat	2015	R	R	R	R
2273	ACN3	M/77	DPT	2015	R	R	R	R
2493	ACN4	M/72	wound	2015	R	R	R	R
2209	ACN5	F/71	DPT	2015	R	R	R	R
3630	ACN6	-	-	2015	R	R	R	R
R4018KM	ACN7	M/77	DPT	2014	S	R	R	R
TKM352	ACN8	-	-	2014	S	R	R	R
R4020 LA	ACN9	M/62	DPT	2014	S	R	R	R
HAR 168	ACN10	-	-	2014	S	R	R	R
SAU 492	ACN11	M/25	urine	2014	S	R	R	R
R1314	ACN12	M/55	sputum	2014	S	S	S	S
Hfnew R697	ACN13	F/83	DPT	2014	S	R	R	R
PFU9	ACN14	-	-	2014	S	R	R	R
R4139NC	ACN15	F/30	DPT	2014	S	R	R	R
ZHIEK1	ACN16	-	-	2014	S	R	R	R
EHR489	ACN17	M/69	DPT	2014	S	R	R	R
R1334	ACN18	M/77	DTA	2014	S	R	R	R
UMMM99	ACN19	-	-	2014	S	R	R	R
I3993JK	ACN20	M/19	Isolate	2014	S	R	R	R
M1561	ACN21	M/66	Lesion	2014	S	R	R	R
R4034MR	ACN22	M/70	DPT	2014	S	R	R	R
R1681	ACN23	F/24	DPT	2014	S	R	R	R
SGR1663	ACN24	M/91	BL	2014	S	R	R	R
R4570	ACN25	-	DPT	2013	R	R	R	R
R1497	ACN26	M/75	DPT	2013	S	R	R	R
B3091	ACN27	F/27	Blood	2013	S	R	R	R
B5259	ACN28	M/23	Blood	2012	R	R	R	R
M3965	ACN29	F/66	Catheter	2012	R	R	R	R
U1357	ACN30	M/67	Urine	2013	S	R	R	R
I1136	ACN31	M/A	Environmental	2013	S	S	S	S
R0069	ACN32	F/47	DPT	2013	S	R	R	R
R2730	ACN33	F/87	DPT	2013	S	R	R	R

Table 3. Antimicrobial susceptibility profile of *E.coli and Enterobacter cloacae* isolates
TIG: Tigecycline, IMP: imipenem, CFP: Cefepime, CTZ: Ceftazdime,
S: susceptible, R: resistant

					Antimicrobial agent			
Isolate #	**Code#**	**Sex/age**	**Origin**	**Year**	**TIG**	**IMP**	**CFP**	**CTZ**
MH2-19	ECOL1	-	Poultry	2016	S	S	R	R
MH2-42	ECOL2		Poultry	2016	S	S	R	R
IMP-791	ECOL3	M/46	Urine	2017	-	R	R	R
IMP-792	ECOL4	M/64	Skin	2017	S	R	R	R
IMP-793	ECOL5	M/82	Urine	2017	-	R	R	R
IMP-797	ECOL6	F/74	Urine	2017	-	R	R	R
B33233	ECOL7	F/11	Blood	2017	S	R	R	R

Table 4. Colistin Antimicrobial Susceptibility testing using (DD) Disc Diffusion, Etest, (BMD) Broth Microdilution, Vitek for *E.coli* and *E.cloacae*

Isolate	**DD (mm)**	**E-test (ug/ml) Colistin sulfate (Polymyxin E)**	**Vitek**	**BMD (ug/ml)**	
				Polymyxin E	**Polymyxin B**
MH2-19	12	2	2	16	32
MH2-42	0	>256	>16	>128	>128
IMP-791	13	1.5	≤0.5	2	4
IMP-792	15	1	≤0.5	128	128
IMP-793	15	0.25	≤0.5	128	128
IMP-797	13	0.5	≤0.5	64	32
IMP-795	14	1	≤0.5	128	32
ATC25922	14	0.25	≤0.5	1	1

Table 5. Colistin Antimicrobial Susceptibility testing using (DD) Disc Diffusion, E-test, (BMD) Broth Microdilution, Vitek for *A. baumannii* isolates

Sample #	Colistin DD(mm)	E-test (ug/ml) Colistin sulfate (Polymyxin E)	Vitek	BMD (ug/ml)	
				Polymyxin E	Polymyxin B
ACN1	0	>256	≥16	>128	>128
ACN2	0	32	4	>128	>128
ACN3	0	>256	≥16	>128	>128
ACN4	12	0.75	≤0.5	4	4
ACN5	0	>256	4	>128	>128
ACN6	0	>256	≥16	>128	>128
ACN7	14	1	≤0.5	4	4
ACN8	15	1	0.5	4	4
ACN9	13	1	0.5	4	4
ACN10	15	0.5	≤0.5	4	8
ACN11	14	0.5	≤0.5	8	32
ACN12	12	1	≤0.5	8	4
ACN13	15	0.75	≤0.5	128	64
ACN14	13	1	≤0.5	8	16
ACN15	10	6	≤0.5	>128	>128
ACN16	11	1	≤0.5	256	256
ACN17	12	1	≤0.5	8	8
ACN18	14	0.75	≤0.5	8	8

	Colistin DD (mm)	E-test (ug/ml) Colistin sulfate (Polymyxin E)	Vitek	BMD (ug/ml)	
				Polymyxin E	Polymyxin B
ACN19	12	0.5	≤0.5	4	4
ACN20	14	0.5	≤0.5	8	4
ACN21	14	0.75	≤0.5	8	8
ACN22	15	0.25	≤0.5	4	4
ACN23	14	1	≤0.5	8	4
ACN24	15	0.5	≤0.5	4	4
ACN25	0	>256	≥16	>128	>128
ACN26	14	1	≤0.5	2	8
ACN27	14	0.5	≤0.5	16	8
ACN28	0	256	≥16	>128	>128
ACN29	0	256	≥16	>128	>128
ACN30	14	0.5	≤0.5	2	4
ACN31	14	0.25	≤0.5	64	32
ACN32	10	12	≤0.5	>128	64
ACN33	14	0.75	≤0.5	8	8

Table 6. Disc Diffusion and MIC Interpretive standards for Colistin

Breakpoints				
	S	**I**	**R**	**Reference**
Disk Diffusion	≥14mm	13mm	≤12mm	[64]
E-Test	≤2ug/ml	-	≥4ug/ml	[65]
Vitek				
Broth Micro Dilution				

Table 7. Agreements and discrepancies between different Antimicrobial susceptibility testing used: DD (Disc Diffusion), BMD (Broth Microdilution), E-test and Vitek.

Agreement-Discrepancies Among the different Antimicrobial Susceptibility testings used in this study	**Number (%) of agreement in isolates**	
	A. baumannii	***E. coli and E.cloacae***
DD-S, E-Test-S, Vietk-S, BMD-S	2 (6)	0
DD-R, E-Test-R, Vietk-R, BMD-R	8 (24)	1
DD-S, E-Test-S, Vietk-S, BMD-R	14 (42)	3
DD-R, E-Test-S, Vietk-S, BMD-R	5 (15)	2
DD-I, E-Test-S, Vietk-S, BMD-R	2 (6)	1
DD-S, E-Test-R, Vietk-S, BMD-R	0	0
DD-S, E-Test-S, Vietk-R, BMD-R	0	0
DD-R, E-Test-R, Vietk-S, BMD-R	2 (6)	0

Table 8. Prevalence of different genes and mutations in *Acinetobacter baumannii*, *E.coli* and *E.cloacae*

Genes		Number (%) of genes in isolates		
		A.baumannii **n=33**	***E.coli*** **n=5**	***E.cloacae*** **n=2**
Lipid Biosynthesis genes	*lpxA*	11(34)	5 (100)	0
	lpxC	32 (97)	5 (100)	1
	lpxD	10 (31)	4 (80)	1
Lipid modifying genes	*pmrA*	22 (67)	5 (100)	0
	pmrB	31(94)	5 (100)	0
	pmrC	33 (100)	5 (100)	0
	eptA	24 (72)	-	-
Porin genes	*OmpA*	33 (100)	-	-
	OmpW	33 (100)	-	-
	OmpC	-	3 (60)	1
	OmpF	-	3 (60)	0
Mutation	*pmrB* T346C	33 (100)	-	-
	pmrB T251A	32(97)	-	-
	pmrB G788T	33 (100)	-	-
	pmrA A24T	0	-	-
Insertion sequence	*ISAba11*	1(3)	0	0
Plasmid encoded gene of resistance	*mcr-1*	0	1 (20)	0
	mcr-2	0	0	0

Table 9. Genetic profile of *Acinetobacter baumannii*
+: positive, -: negative

	Sample#	Col	*mcr-1*	*lpxD*	*lpxA*	*lpxC*	*pmrA*	*pmrB*	*pmrC/ eptA*	$OmpA_{Ab}$	*OmpW*
1	ACN1	R	-	-	-	_	_	_	+/+	+	+
2	ACN2	R		+		+	_	_	+/+		
3	ACN3	R		+		+	_	+	+/+		
4	ACN4	R		+		+	_	+	+/+		
5	ACN5	R		+		+	_	+	+/+		
7	ACN6	R		+		+	_	+	+/+		
8	ACN7	R		+		+	_	+	+/+		
9	ACN8	R		+		+	_	+	+/+		
10	ACN9	R		+		+	_	+	+/+		
11	ACN10	R		+		+	+	+	+/+		
12	ACN11	R		+		+	+	+	+/+		
13	ACN12	R				+	+	+	+/+		
14	ACN13	R				+	_	+	+/_		
15	ACN14	R				+	_	+	+/+		
16	ACN15	R				+	+	+	+/_		
17	ACN16	R				+	+	+	+/+		
18	ACN17	R				+	+	+	+/_		
19	ACN18	R				+	+	+	+/+		
20	ACN19	R				+	+	+	+/+		
21	ACN20	R				+	+	+	+/_		
22	ACN21	R				+	+	+	+/_		
23	ACN22	R		-	+	+	+	+	+/+		
24	ACN23	R			+	+	+	+	+/+		
25	ACN24	R			+	+	+	+	+/_		
26	ACN25	R			+	+	+	+	+/+		
27	ACN26	S			-	+	+	+	+/_		
28	ACN27	R			+	+	+	+	+/_		
29	ACN28	R			+	+	+	+	+/-		
30	ACN29	R			+	+	+	+	+/+		
31	ACN30	S			+	+	+	+	+/+		
33	ACN31	R			+	+	+	+	+/+		
34	ACN32	R			+	+	+	+	+/+		
35	ACN33	R			+	+	+	+	+/+		

Table 10. Insertion sequence and Mutations in *Acinetobacter baumannii* resistant and susceptible isolates

+: positive, -: negative, MIC in µg/mL, Col: Colistin

	Sample #	Col	*ISAba11*	*pmrAA24T*	*pmrBG788T*	*pmrB T346C*	*pmrB T521A*
1	ACN1	R	–	–	+	+	+
2	ACN2	R					
3	ACN3	R					
4	ACN4	R					
5	ACN5	R					
7	ACN6	R					
8	ACN7	R					
9	ACN8	R					
10	ACN9	R					
11	ACN10	R					
12	ACN11	R					
13	ACN12	R	+				
14	ACN13	R	–				
15	ACN14	R					
16	ACN15	R					
17	ACN16	R					
18	ACN17	R					
19	ACN18	R					
20	ACN19	R					
21	ACN20	R					
22	ACN21	R					
23	ACN22	R					
24	ACN23	R					
25	ACN24	R					
26	ACN25	R					
27	ACN26	S					
28	ACN27	R					
29	ACN28	R					
30	ACN29	R					
31	ACN30	S					
33	ACN31	R					
34	ACN32	R					
35	ACN33	R					–

Table 11. Genetic profile of *E. coli* isolates and *Enterobacter cloacae ECOL7 and ECOL5*
+: positive, -: negative

	Sample #	Col	*mcr-1*	*mcr-2*	*lpxA*	*lpxD*	*lpxC*	*pmrA*	*pmrB*	*pmrC*	*Isaba11*	*OmpC*	*OmpF*
58	ECOL1	R	+	_	+	_	+	+	+	+	_	_	+
59	ECOL2	R	_	_	_	_	+	_	_	_	_	_	_
IMP791	ECOL3	S	_	_	+	+	+	+	+	+	_	+	+
IMP792	ECOL4	R	_	_	+	+	+	+	+	+	_	+	+
IMP793	ECOL5	R	_	_	_	+	_	_	_	_	_	+	_
IMP797	ECOL6	R	_	_	+	+	+	+	+	+	_	+	_
B33233	ECOL7	R	_	_	+	+	+	+	+	+	_	_	_

Table 12. Colistin resistance by different antimicrobial susceptibility testing versus gene distribution in *A. baumannii*, *E.coli* and *E.cloacae* S: Sensitive I: Intermediate R: Resistant

Antimicrobial Susceptibility testing technique		**Number (Percentage) of Isolates**	**Distribution of Genes Number (Percentage) for *A. baumannii (n=33)***						
			lpxD	***lpxA***	***lpxC***	***pmrA***	***pmrB***	***pmrC***	***eptA***
Disc Diffusion	**S**	16 (48.5)	4 (25)	8 (50)	16(100)	13 (81)	16(100)	16 (100)	9(56)
	I	2 (6)	1 (50)	0	2 (100)	0	2 (100)	2 (100)	2 (100)
	R	15 (45.5)	5 (33)	4 (26)	14 (93)	9 (60)	13 (86)	15 (100)	11 (73)
E-Test	**S**	23 (70)	6 (26)	7 (30)	23 (100)	17 (74)	23(100)	23 (100)	16 (69)
	R	10 (30)	4 (40)	3 (30)	9 (90)	4 (40)	8 (80)	10 (100)	7 (70)
Vitek	**S**	25 (76)	6 (24)	8 (32)	25 (100)	19 (76)	25 (100)	25 (100)	17 (68)
	R	8 (24)	4 (50)	3 (37)	7 (87)	3 (37)	5 (62)	8 (100)	7 (87)
Broth Micro Dilution	**S**	2 (6)	0	1 (50)	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)
	R	31 (94)	10 (32)	10 (32)	30 (97)	20 (64)	29 (93)	31 (100)	23 (74)

Table 13. Colistin resistance by different antimicrobial susceptibility testing versus gene distribution in *E.coli* and *E.cloacae* S: Sensitive I: Intermediate R: Resistant

Antimicrobial Susceptibility testing technique		No. of Isolates	Distribution of Genes (Number and percentage) for *E.coli* (n=5)							
			lpxD	***lpxA***	***lpxC***	***pmrA***	***pmrB***	***pmrC***	***mcr-1***	***mcr-2***
Disc Diffusion	**S**	2	2	2	2	2	2	2	0	0
	I	2	2	2	2	2	2	2	0	0
	R	1	0	1	2	1	1	1	1	0
E-Test	**S**	5	5	5	5	5	5	5	1	0
	R	0	0	0	0	0	0	0	0	0
Vitek	**S**	5	5	5	5	5	5	5	1	0
	R	0	0	0	0	0	0	0	0	0
Broth Micro Dilution	**S**	1	1	1	1	1	1	1	0	0
	R	4	4	4	5	4	4	4	1	0

Table 14. Prevalence of *mcr-1* in *E.coli* in selected countries from different regions

Country		Resistance genes	Isolate	Origin	Reference
Middle East	Bahrain (N=2)	5.3% *mcr-1*	*E.coli*	Human Isolate	[66]
	KSA (N=1)		*E.coli*		
	UAE (N=1)		*E.coli*		
Europe	Germany	79.8% *mcr-1*	*E.coli*	Animal origin	[67]
	Belgium (N= 13)	12.4% *mcr-1*			[52]
	France (N=517)	21% *mcr-1*			[68]
			E.coli	Human origin	[68]
Latin America	South America (N=515)	0.3% *mcr-1*	*E.coli*	Human and Animal origin	[69]
Southeast Asia	Thailand (N=19)	63% *mcr-1*	*E.coli*	Human isolate	[70]
	Vietnam (N=200)	59.4% *mcr-1*	*E.coli*	Animal origin	[71]
		20.6% *mcr-1*	*E.coli*	Human isolate	
	China (N=523)	15% *mcr-1*	*E.coli*	Animal origin	[49]
	China (N=804)	21% *mcr-1*	*E.coli*	Animal origin	
	China (N=1322)	1% mcr-1	*E.coli*	patient	
Africa	Egypt (N=185)	*1 isolate mcr-1*	*E.coli*	Animal origin	[72]
	Tunisia (N=512)	20% *mcr-1*	*E.coli*	Animal origin	[73]
		17% *mcr-1*	*E.coli*	Animal origin	
		83% *mcr-1*	*E.coli*	Animal origin	
USA		*1 isolate mcr-1*	*E.coli*	Animal origin	[74]
		1 isolate mcr-1	*E.coli*	Human isolate	[75]

Table 15. Comparative findings colistin resistance genes and mutations in *A. baumannii* reported in countries from different region Ø: No mutation

	Country	*pmrA*	*pmrB*	*pmrC*	*lpxA*	*lpxC*	*lpxD*	Isolate	Origin	Ref
Our study	Lebanon (N=33)	69%	*94% (97% S17R 100%, R263L and Y116H)*	100%	34% *ISAba11*	97%	31%	*A. baumannii*	Human Isolate	-
Middle East	Iran (N=100)	70%	30%	-	-	-	-	*A. baumannii*	Human Isolate	[76]
Europe	Greece (N=86)	*100 %G54E*	-	*100% pmrCR 109H*	*100%Y131H*	*100% C120R and N287D*	*100% E117K*	*A. baumannii*	Human isolate	[77]
	Spain (N=9)	22% R81 22% V161		-	-	-	-	*E.coli and S.*enetrica	Animal origin	[78]
USA	USA (N=14)	*43% E8D*	*14% S17R, 36% T232I, and 14% Y116H*	*36%*	-	-	-	*A. baumannii*	Human isolate	[46]
Latin America	Brazil (N=2)	-	*100%Tre28 and 45Glu*	-	*100% ISAba125*	-	-	*A. baumannii*	Human Isolate	[79]
Australia	Australia (N=12)	-	-	-	*ISAba11 H159D, D233,G68D, D130,Q72K,H121*	*ISAba11 P30H, D45,T285, P30L*	*K317*	*A .baumannii*	Human isolate	[48]
Southeast Asia	Malaysia (N=14)	100 % P120H	Ø	Ø	Ø	100% *K141R , S158R*	100% *S102T,V141I,R173G G186S, E50D*	*A. baumannii*	Human isolate	[80]

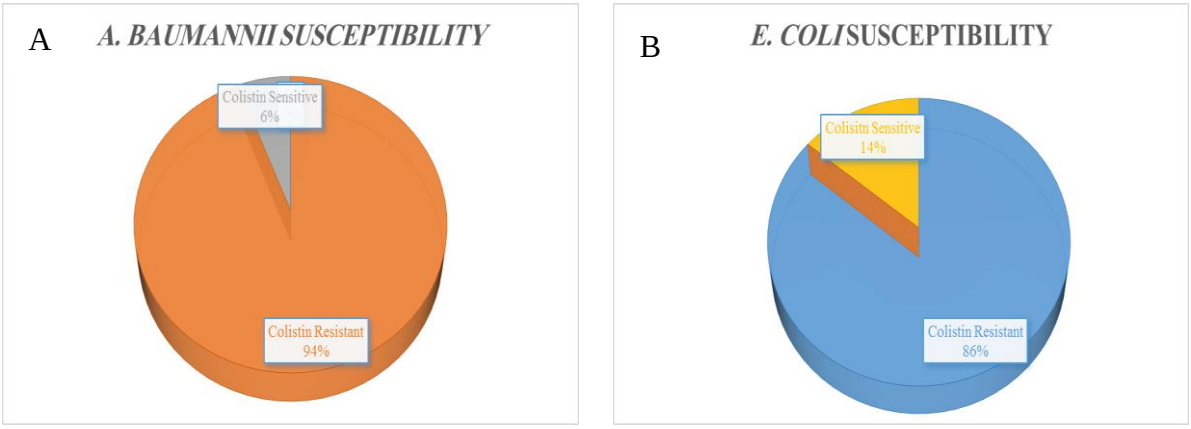

Fig. 1 Pie Chart showing the percentage of resistant and sensitive *A. baumannii* (A) and *E.coli and E. cloacae* isolates (B)

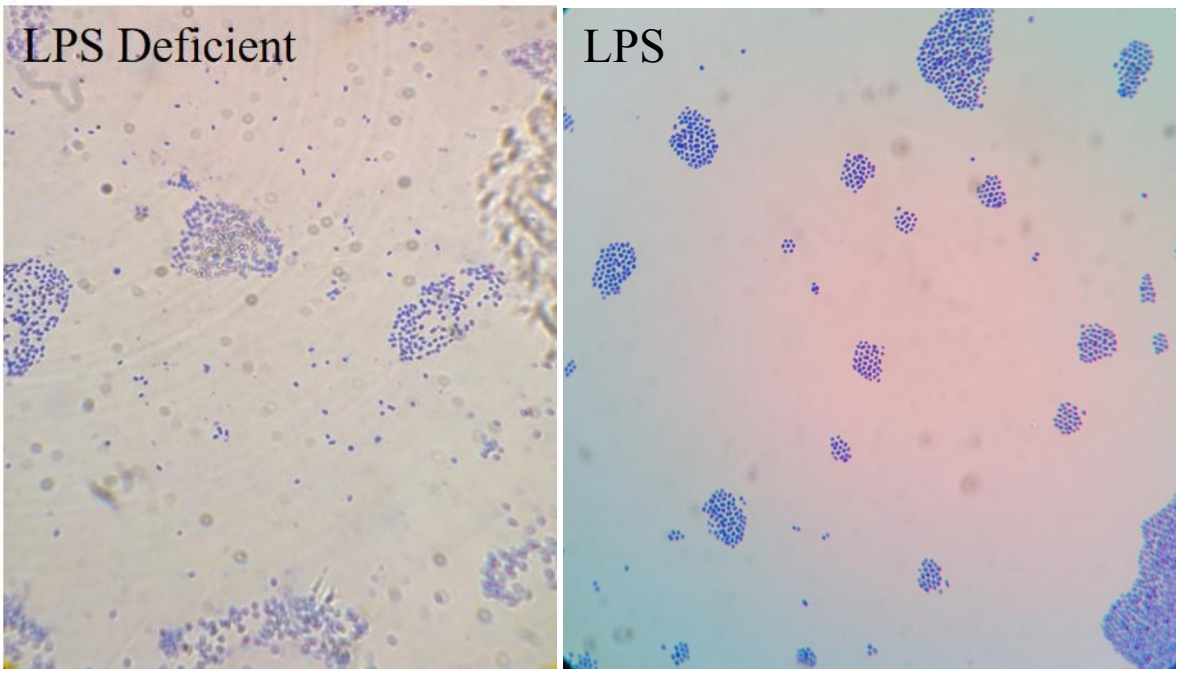

Fig 2. Visualization of the capsule *for A. baumannii* under light microscopy LPS deficient ACN1 and *A. baumannii* possessing LPS ACN33 magnification x100

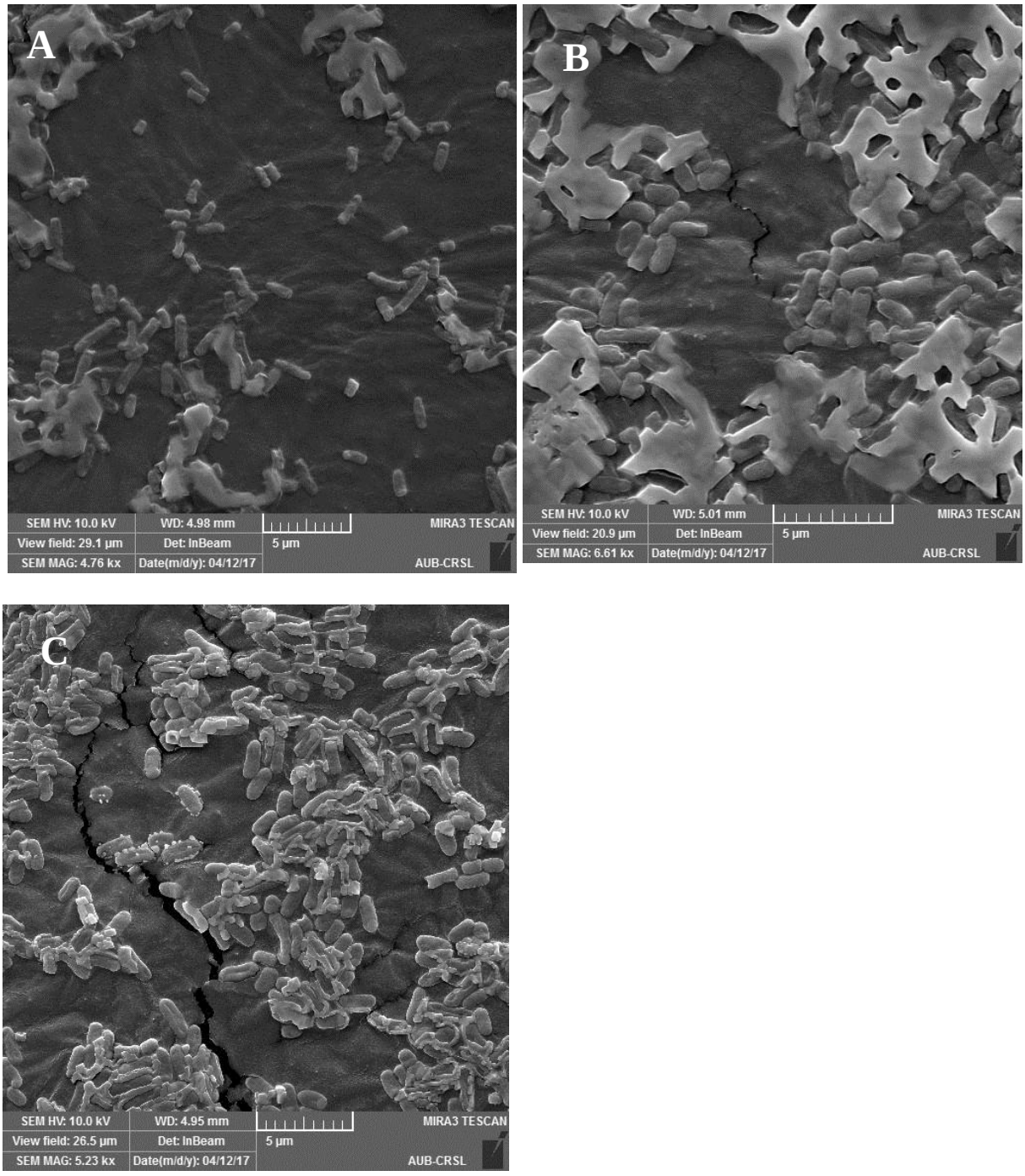

Fig 3. SEM image for *E.coli* colistin sensitive ATCC25992 (A), untreated MCR-1 *E.coli* (B) treated MCR-1 *E.coli* (C) platinum coated

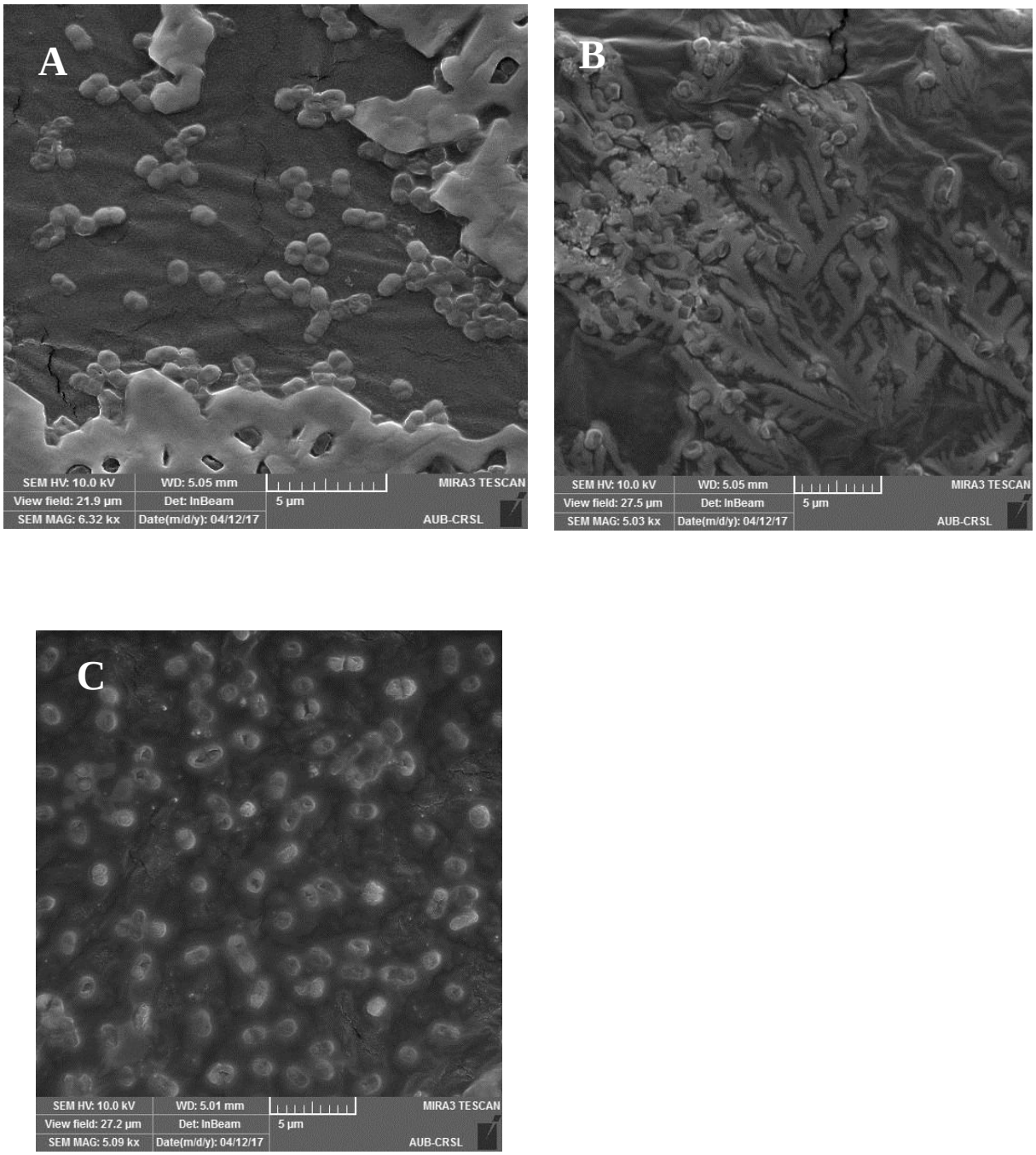

Fig 4. SEM for colistin Sensitive *Acinetobacter baumannii* (A) Untreated Colistin Resistant *Acinetobacter baumannii* (B) Treated Colistin Resistant *Acinetobacter baumannii* (C)

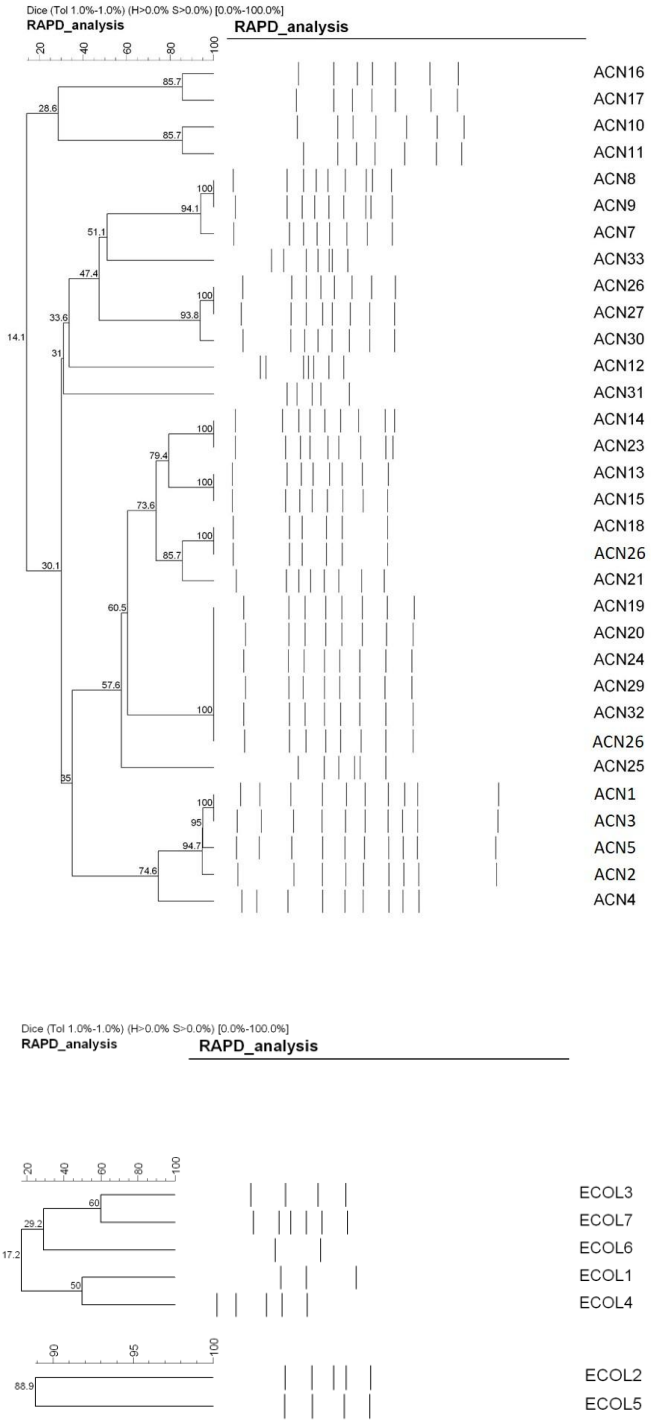

Fig 5. Dendogram obtained from RAPD anlysis for *A.baumannii, E. coli and E. cloacae*

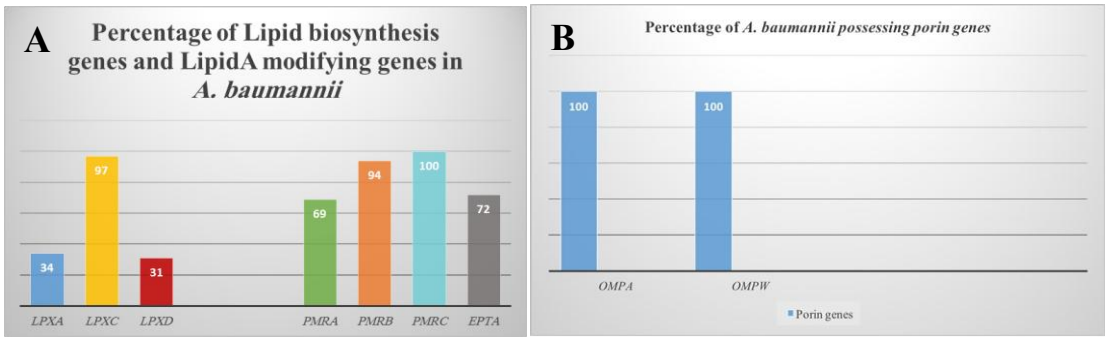

Fig 6. Perecentage of Lipid biosynthesis genes, Lipid A modifying genes(A) and Porin genes in *A. baumannii* (B)

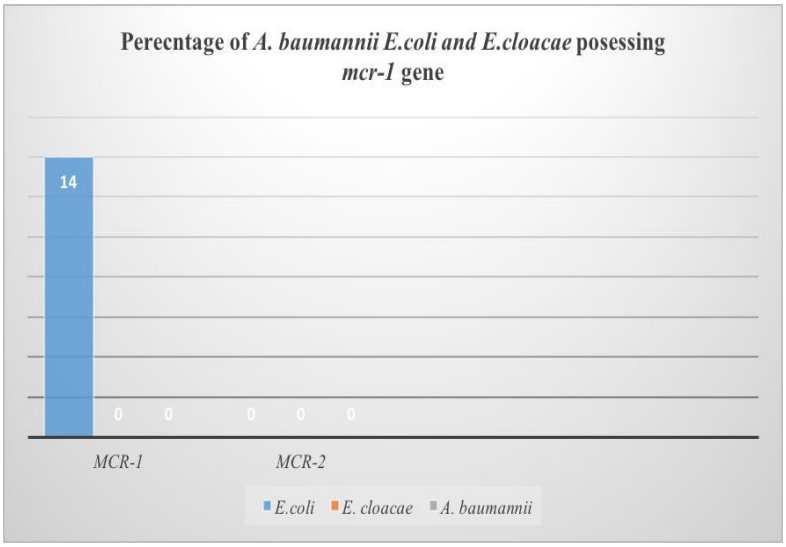

Fig 7. Percentage of *mcr-1* and *mcr-2* gene in *E.coli*, *E. cloacae*, and *A. baumannii*

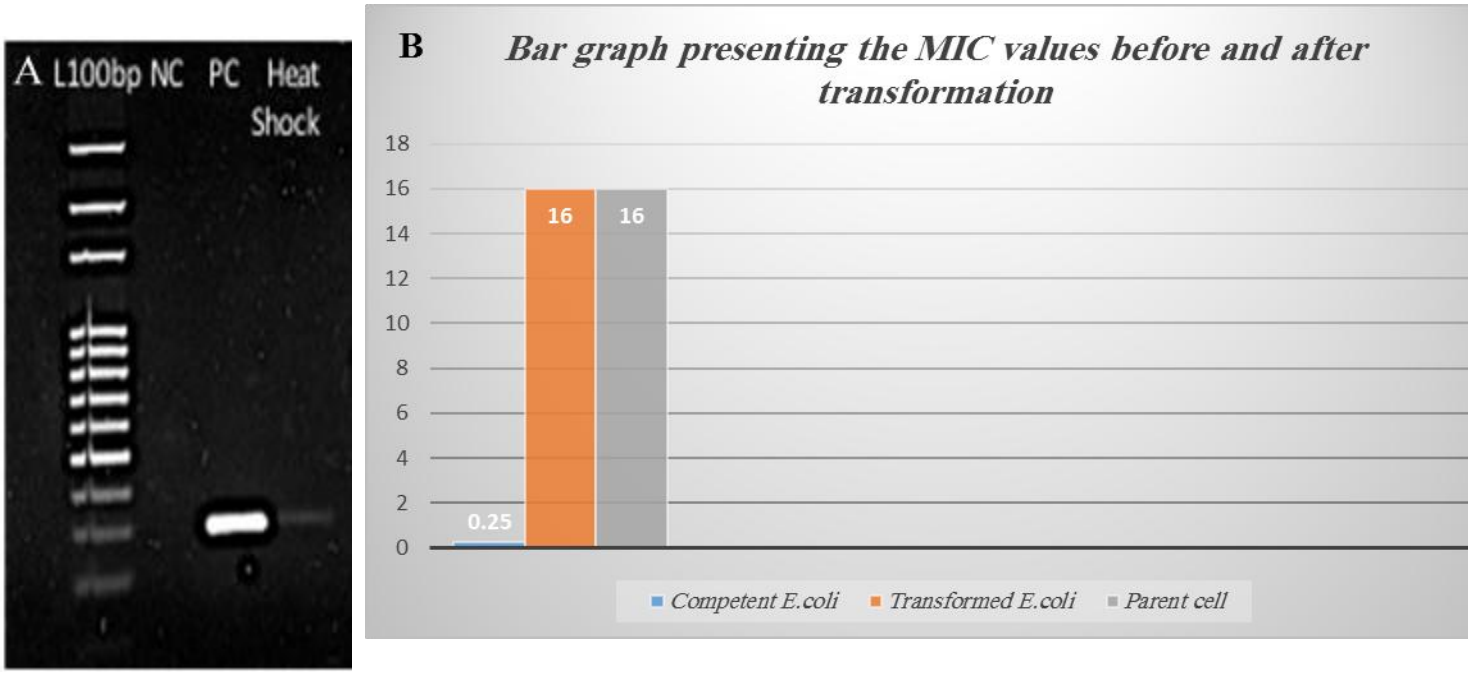

Fig 8. Gel electrophoresis results for the extracted plasmid from transformed *E.coli* (A) Bar graph presenting MIC values before and after transformation (B)

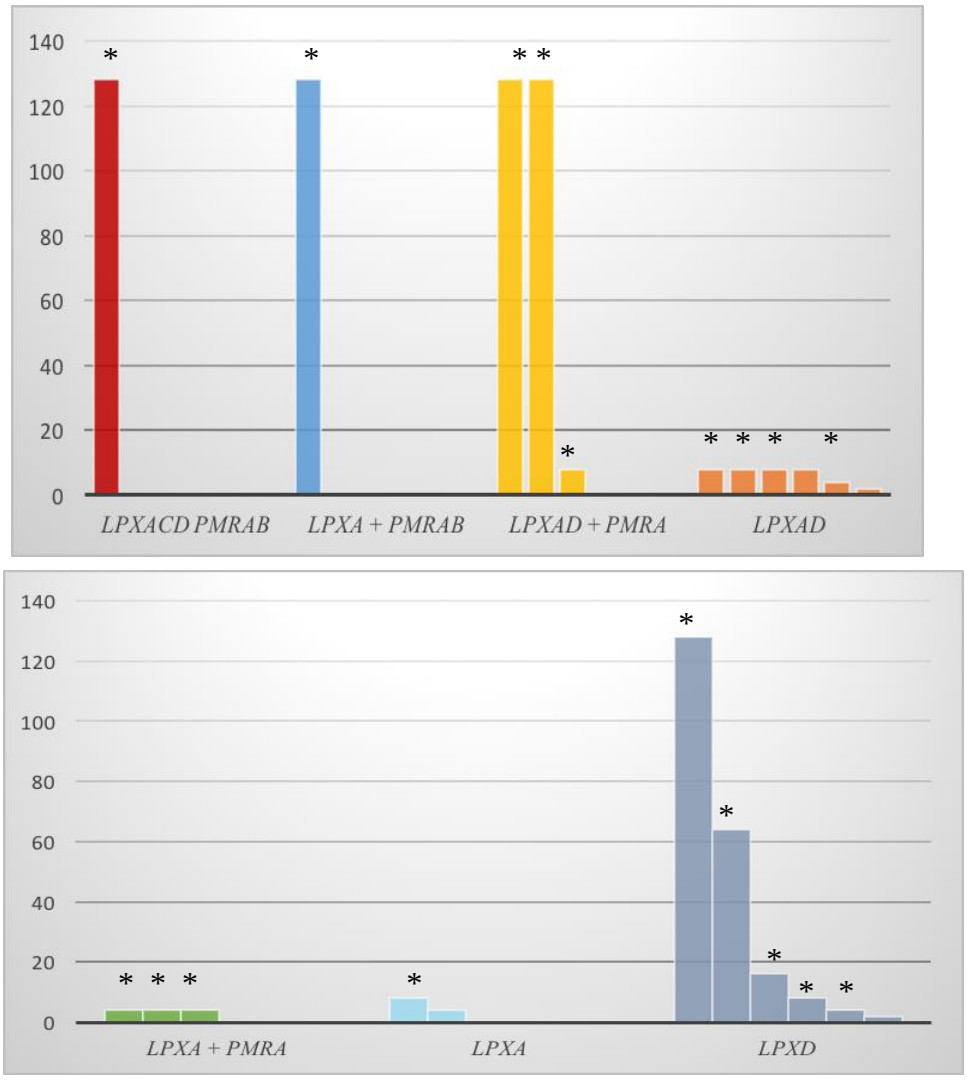

Fig 9. Relationship between minimum inhibitory concentration in µg/mL and loss of genes in *A. baumannii* isolates *: presence of *eptA* gene

CHAPTER V

DISCUSSION

Generally, the bacteria employ several mechanisms to protect itself from cationic antimicrobials, such as colistin. These are alterations in their lipopolysaccharide by the Two-component system pmrAB and target modification or LPS loss [49,81]. Colistin resistance was always thought to be chromosomally encoded until a surveillance project in China on pigs identified a novel mechanism of resistance, plasmid mediated colistin resistance *mcr-1*. Subsequently, by the year 2016 the *mcr-1* harboring *E.coli* were isolated from animal and human isolates and had spread to almost all continents Asia, Europe, Africa, and America[82].

The shortage of new antimicrobials to treat multi-drug resistant Gram-negative bacteria has led to the reuse of colistin, a polymixin. The lack of information regarding polymyxins' pharmacokinetics/pharmacodynamics hindered the ability of practitioners to optimize colistin doses in humans, especially those with renal failure [83]. Moreover, Colistin methanosulfate was used for clinical treatment while other forms (polymyxin E and polymyxin B) for susceptibility testing. All these factors contributed to the development of colistin resistance [83,84]. Colistin remain the last treatment of choice for multi-drug resistant Gram-negative infections. However, MIC trends of colistin resistance is increasing worldwide, underlying the vital need for a suitable susceptibility testing [58]. In Lebanon and at the AUBMC, colistin resistance is increasing from 2012 to 2016. Various studies showed E-test method and Disc diffusion technique to produce excessive rates of very major errors (VMEs) [58]. This is attributed to the inability of large

polymyxin molecules to diffuse through the agar. As for the automated system Vitek, controversial results have been reported about its reliability in determining colistin resistance. Vitek showed low sensitivity in terms of detecting heteroresistant subpopulations. Therefore, it can be used to determine colistin susceptibility in isolates that do not show heteroresistance. [85,86,87]. In this study DD, E-test, BMD and the automated system Vitek were used to determine the colistin susceptibility profile of our isolates. Disc diffusion and E-test methods are the most frequently used techniques in clinical laboratories. Many concerns have questioned the reliability of E-test and Disc diffusion in determining colistin resistance due to the drug's poor ability to diffuse in the agar [56]. Supporting this, our data revealed E-test method on MH agar to give lower MIC values in comparison with BMD. Thus, providing false susceptibility results but no false resistant results [58]. Similarly for DD, false susceptible results were found and our data provided minor concordance between DD and BMD. Furthermore, colistin resistant results by DD were true [88]. Previous studies have shown Vitek for *A.baumannii* colistin susceptibility testing to show good performance however those studies used colistin susceptible *A. baumannii* [85]. Our data revealed Vitek to detect high level of resistance but failed at low level of resistance due to its poor sensitivity especially in detecting heteroresistance [86]. Therefore, the dilution methods remain the gold standard reference method for determining colistin resistance.

RAPD analysis showed genomic diversity in *A. baumanii* and *E. coli* isolates. On the other hand, clusters showing similar patterns arise from common progenitors.

Colistin resistance, as already been mentioned, occurs due to mutations in *lpx* and/or *pmr* genes all contributing to bacterial cell membrane remodeling (Table 15). *pmrB* gene was mostly found to be mutated in different regions of the world. These mutations were determined to be in the periplasmic and the histidine kinase domains influencing the interaction between *pmrA* and *pmrB,* or leading to a gain of function in *pmrB.* Regarding LPS mutants globally, number of mutations were observed ranging from point mutations to deletions and insertion sequences. One of these mutations is P30 in *lpxC* and and another, H159 in *lpxA,* were shown to be critical for enzyme activity and involved in substrate binding [89]. Our data revealed that all our isolates harbor *pmrB S17R, Y116H, Y116H* mutations contributing to the colistin resistant phenotype. The higher percentage, almost double, in the prevalence of *lpxA, lpxC* and *pmrA, pmrB, pmrC* genes in colistin susceptible isolates in comparison to colistin resistant isolates by different susceptibility tests highlights their key role in contributing to the colistin resistant phenotype [48,81]. Total loss of LPS and/or *pmrB* mutations, overexpression of *pmrAB* and expression of *pmrC* in *A. baumannii* lead to colistin resistance [48,90,91]. Our data was the first to tackle all common mechanisms in *A. baumannii* and *E.coli* and showed combination of mechanisms contributing to colistin resistance (Fig. 10). This study demonstrated a relationship between *pmrB, lpxC* along with, *lpxA* together with *lpxD* loss and minimum inhibitory concentrations (MIC). A prominent increase in MIC values was observed particularly in *lpxC* and *pmrB* loss [91]. For example, total loss of LPS via loss of rate limiting enzyme (*lpxC*), *lpxA, lpxD*) *and* mutations in *pmrA pmrB* lead to high level of resistance (128 μg/mL) as seen in one isolate; ACN1.

Loss of *lpxA* and/or *lpxD* contributed to MIC degrees of 4 and 8 μg/mL. Emiola *et al.* suggested the possibility of *lpxD* to substitute *lpxA* in Lipid A biosynthesis [92]. We postulate that the high level of resistance resulted due to the presence of the *eptA* gene overexpression of *pmrAB* and cross-talk between two-component systems that transduce stress signals from the environment and allows bacterial adaptation [93,94]. This allows the regulated genes to be affected by more than one signal. One of the two-component systems, PhoPQ system was demonstrated to positively activate pmrAB in response to cationic peptides like colistin [43,95]. This was studied extensively in *Pseudomonas aeruginosa, Salmonella* Typhimurium, *Klebsiella pneumoniae* however to date no study have tackled this promising system in *A. baumannii* [96].

The most common mutations in *A. baumannii* that have been attributed to colistin resistance are: *pmrA A24T*, *pmrB T521A, pmrB T346C* and *pmrB G788T* [45,46]. *pmrA E8D,* lays in pmrA receiver domain that accepts a phosphate from the kinase and is important for DNA binding and constitutive gene expression, was not found in any of our clinical isolates. However, PCR analysis shows a further mutation that needs to be investigated by full genome sequencing. On the other hand, *pmrA* contributed to increase in resistance seen in isolates ACN1,2,7,8,9,13,14and 16. As for *pmrB*, the substitution mutations *pmrB T521A* (amino acid S17R), and *pmrB T346C* (amino acid Y116H) occurred at the periplasmic domain of *pmrB. pmrB G788T* (amino acid R263L) occurred outside the histidine-kinase domain and was thought to influence the interaction between PmrA and PmrB. *pmrB T346C and pmrB G788T* were found in all our isolates even in colistin sensitive ACN30 and ACN26. Furthermore, these two isolates were shown to emerge form

the same progenitor by RAPD analysis. In consensus with Nhu *et. al* and Snitkin *et al., A. baumannii* have the ability to induce other mutations that overrides colistin resistant phenotype [47,95]. Therefore, in order for *A. baumannii* to establish colistin resistance, it needs to accumulate further mutations [47]. *Acinetobacter* with various mutations in *pmrA* and *pmrB* could coexist in the same patient and go undetected.

Our data revealed that the presence of an additional phosphoethanolamine transferase *eptA,* adds phosphoethanolamine to the core-Lipid A, in *A. baumannii* has a tendency of developing colistin resistance more promptly than the ones that do not. *eptA* lies 900 bp upstream from a putative integrase gene and exists at the extremities of prophages [46]. This provides evidence that *eptA* gene may have been duplicated within the ancestral strain or have been horizontally acquired. ACN11 and ACN24 both have the same genetic profile however, differ in their MIC 8 μg/mL and 4 μg/mL respectively. This increase in MIC is attributed to the fact that ACN11 possess an additional phosphoethanloamine transferase that is encoded by an *eptA* gene.

The insertion sequence *ISAba11* is replicative, mobile, and was found to be part of a transposon Tn*6021* [48]. This 1.1 kb sequence is flanked by two inverted repeats and was shown to encode a transposase. ACN12 was the only isolate that was shown to harbor *ISAba11* sequence and confer resistance. Moreover, ACN12 was also devoid of *lpxA* and *lpxD* concurrently to what was identified by Moffat *et al.* who showed by sequence analysis the presence of the insertion sequence *ISAba11* in either *lpxA* or *lpxC* genes this contributed to high levels of MIC.

LPS is a crucial part of Gram-negative bacterial membrane. The loss of LPS raised concern about the ability for ACN1 to survive. Capsule staining showed an extensive halo

in ACN1 in comparison with ACN33 having LPS and bacteria more clustered. *A. baumannii* hyperproduction of capsular exopolysaccharide is a reversible mechanism and it is associated with increased resistance to the inducing antibiotic [97]. This supports Henry *et. al* who reported that total loss of LPS alters *A. baumannii* bacterial membrane by upregulating genes involved in envelope and membrane biosynthesis such as phospholipids and poly-β-1,6-*N*-acetylglucosamine (PNAG). Similar mechanism has been identified in intrinsically colistin resistant *N. meningitidis* [97,98].

Adaptive resistance such as efflux pumps and porins are transitory in nature and bacteria usually revert back to their normal form upon the removal of these inducing agent [35]. $OmpA_{Ab}$ is the most abundant porin in *A. baumannii* [27,28,99]. All our isolates were shown to harbor this gene. Chopra *et al.* showed that OmpW porin to be more abundant in multi-drug resistant *A. baumannii.* Several studies have reported the under-expression of OmpW in colistin resistance [29,30]. Colistin was demonstrated to interact with OmpW in *A. baumannii*. Upon screening, we noted that all our isolates harbored the *OmpW* gene. Catel-Ferreira *et al.* displayed that the loss of OmpW expression had no effect on colistin susceptibility in *A. baumannii* [30]. Therefore, colistin resistance in our isolates were not due to porin loss.

Colistin resistance in our *E. coli* isolates was neither due to *pmrA*/*pmrB* loss nor to LPS loss opposing to *E. cloacae* isolates. We postulate that resistance in our clinical isolates was either due to upregulation of *pmrAB* or cationic sugar 4-amino-4-deoxy-l-arabinose (L-Ara4N) addition which is not found in *A. baumannii*. It is worthy to note that colistin resistance mediated by LPS modification in *E.coli* is mainly attributed to the *arnT* operon that adds L-Ara4N to lipid A, followed by *pmrAB* operon.[43,100,101].

Humans may acquire antimicrobial resistance from animal origin through food, animal contact or the environment [82]. Concerning plasmid-mediated colistin resistance by MCR-1 and MCR-2 plasmids, only one isolate of poultry origin carried the *mcr-1* gene and non of our isolates carried the *mcr-2* gene. Transformation experiments were done to demonstrate the ability of MCR-1 to mobilize and to solely cause polymyxin resistance. Upon transformation of MCR-1 plasmid to colistin susceptible *E.coli* by heat shock, a 16-fold increase in MIC was seen in the transformed cell similarly to findings of Lui *et al.* [49,103]. The MIC value upon transfer highly increased to be equivalent to the parent cell (16ug/ml).

The loss of porin genes increased the level of resistance. This was seen in ECOL2,5,6 and ECOL7. Acquired resistance such as porin loss and efflux pump have a direct consequence on resistance by lowering the availability of the drug, thus enhancing secondary intrinsic resistance [35]. Studies have demonstrated that mutations in porin genes can occur during therapeutic treatment [102]. Such adaptive resistance was revealed in our *E.coli* isolates but not in *A. baumannii*.

Scanning electron microscopy (SEM) was used to directly visualize the differences in cell morphology especially upon colistin treatment. No study to date approached the role of MCR-1 on *E.coli* cell membrane. SEM displayed slight cell morphological changes between colistin sensitive and resistant isolates as well as minor perturbations upon colistin

treatment. This manifested the action of colistin on the bacterial membrane [103,104]. The roughened spherical features observed are due to cellular leakage [105].

As a conclusion, colistin resistance in Gram-negative bacteria is mediated by several mechanisms targeting bacterial cell membrane remodeling. Molecular characteristics of Gram-negative isolates in this study demonstrated that such resistance is mediated by LPS loss and/or modifications primarily in *A. baumannii* and *E.cloacae*. Colistin resistance phenotype occurs due to the accumulation of mutations, various signal cues from different Two-component systems and intrinsic factors such as the presence of *eptA in A. baumannii.* Despite the great deal of information on colistin resistance, many underlying mechanisms need to be investigated in order to construct an effective combination therapy. The identification of the mobile colistin resistance gene *mcr-1*, its ability to disseminate worldwide from animals to humans and to solely induce colistin resistance imposes a global threat to public health globally.

Future approaches should involve full genome sequencing to identify novel mutations in clinical isolates. Moreover, lab induced mutations should be constructed in order to identify and track mutations contributing to the colistin resistant phenotype. This will allow pharmaceutical companies to modify colistin drug in a way to overcome this resistance. Furthermore, an effective combination therapy targeting different mechanisms of resistance should be implemented. On the other hand, surveillance on colistin utilization in treatment and in animal feeds should be made thus limiting further dissemination and increase in colistin resistance.

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