Adaptation of *Acinetobacter baumannii* to Colistin Exposure: *in vivo* and *in vitro* Experience

by

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ABSTRACT

Acinetobacter baumannii (A.baumannii) is one of the emerging pathogens which causes severe infections with high mortality. Multidrug-resistant (MDR) and colistin-resistant *A.baumannii* spread worldwide especially in healthcare centers. *A.baumannii* has two genetic mechanisms of colistin resistance: Complete loss of LPS as a result of mutations in *lpx* genes and point mutations in *pmrCAB* operon. Both mechanisms cause structural changes and eventually prevent colistin binding to cell wall. However, triggering factors and evolutionary mechanisms of colistin resistance are still not clearly understood. In this study, we aimed to mimic induction of colistin resistance by colistin exposure, follow-up the progression of resistance and compare *in vitro* results with *in vivo* resistance identified in an isolate from a patient.

A 35-years old female patient admitted in VKV American Hospital (Istanbul) intensive care unit between February and March 2016 was chosen for the study. A total of four colistin susceptible and one colistin resistant *A.baumannii* isolates were collected. Patient's clinical data and outcome were recorded. Daily serial passages of susceptible isolates in presence of colistin at 1 mg/L concentration were performed to mimic development of colistin resistance *in vitro* by colistin exposure. Minimum inhibitory concentrations (MICs) were measured by broth microdilution. The colistin-resistant isolate and certain generations of *in vitro* experiment were chosen for molecular analyses. The *pmrCAB* complete operon and *lpxA*, *lpxC,* and *lpxD* genes were sequenced. The expression levels of *pmrC*, *pmrA*, and *pmrB* were studied by qRT-PCR. The patient's data and the results of molecular tests were compared.

The *pmrA, pmrB* and *pmrC* genes were 1.6, 1.74 and 1.72 times overexpressed in colistin resistant clinical isolate than susceptible one. Colistin resistance was identified in the patient at 25th day of the colistin therapy. In 38 serial passages of four susceptible isolates, colistin MIC values were above breakpoint level of 2 mg/L after first passage. At the 26th generation of experiment, *pmrC* expressions of all isolates reached to a peak level (2.11; 29.65-fold). Moreover, in 2 of the four isolates *pmrA* (1.97 and 8.54-fold) and *pmrB* (2.31 and 11.24-fold) expressions were at the highest level in the same generation. We also detected multiple insertions in *pmrA, pmrB, pmrC, lpxA, lpxC* and *lpxD* genes.

In conclusion, upregulation of *pmrC*, *pmrA*, and *pmrB* in combination with insertions in these genes and *lpx* genes may trigger development of colistin resistance. The highest *pmrCAB* expressions after 26th day of exposure and isolation of resistant strain from the patient at 25th day of colistin therapy suggested us that long term therapy is required for development of colistin resistance in *A.baumannii*. Therefore, duration of colistin use and combined therapy options should be considered and controlled properly during colistin therapy.

ÖZETÇE

Acinetobacter baumannii (A.baumannii) ciddi enfeksiyonlara ve yüksek ölüm oranlarına neden olan patojenlerden biridir. Çoklu antibiyotik dirençli ve kolistin dirençli *A.baumannii* dünyada özellikle hastanelerde hızla yayılmaktadır. *A.baumannii*'de kolistin direncine neden olan 2 farklı mekanizma vardır: *lpx* genlerinde meydana gelen mutasyonlar ve *pmrCAB* operonunda meydana gelen nokta mutasyonları. İki mekanizma da hücre duvarının yapısında değişimlere neden olarak kolistinin bağlanmasını engellemektedir. Ancak tetikleyici faktörler ve colistin direncinin evrimsel mekanizmaları hala açıklığa kavuşmamıştır. Biz bu çalışmada kolistin kullanımına bağlı kolistin direnci gelişimini laboratuvar ortamında taklit ederek, direncin ilerlemesini takip etmeyi ve in vitro elde edilen sonuçları hastada *in vivo* gelişen direnç ile karşılaştırmayı hedefledik.

VKV Amerikan Hastanesi (İstanbul) yoğun bakım ünitesinde Şubat – Mart 2016 döneminde yatan 35 yaşında kadın bir hasta çalışmaya dâhil edildi. Toplam dört kolistin duyarlı ve bir kolistin dirençli izolat toplandı. Hastanın klinik verileri ve tedavi sonuçları kaydedildi. Kolistin maruziyetine bağlı kolistin direnci gelişiminin taklidi için duyarlı izolatların 1 mg/L kolistin varlığında günlük seri pasajları yapıldı. Nesillerin minimum inhibitör konsantrasyon (MİK) değerleri sıvı mikrodilüsyon yöntemi ile ölçüldü. Kolistin dirençli klinik izolat ve *in vitro* deneylerden belirli nesiller moleküler analizler için seçildi. *pmrCAB* operonu, *lpxA*, *lpxC* ve *lpxD* genleri sekanslandı. *pmrC*, *pmrA*, and *pmrB* genlerinin ekspresyonları qRT-PCR ile tespit edildi. Hastanın verileri ve moleküler testlerin sonuçları karşılaştırıldı.

pmrA, pmrB ve pmrC genlerinin ekspresyonu kolistin dirençli klinik izolatta kolistin duyarlıya göre sırasıyla 1.6, 1.74 ve 1.72 kat fazla bulundu. Kolistin direnci hastada kolistin tedavisinin 25. günü tespit edildi. Dört duyarlı örneğin 38 seri pasajında, ilk pasajdan sonra kolistin MİK değerleri sınır değeri olan 2 mg/L'nin üzerindeydi. Deneyin 26. jenerasyonunda bütün izolatların *pmrC* ekspresyonu en yüksek değerine ulaştı (2.11-; 29.65 kat). Aynı jenerasyonda dört izolatın ikisinde *pmrA* (1.97 ve 8.54 kat) ve *pmrB* (2.31 ve 11.24 kat) ekspresyonları en yüksek değerlerine ulaştı. Ayrıca *pmrA, pmrB, pmrC, lpxA, lpxC* ve *lpxD* genlerinde çok sayıda insersiyon ttespit edildi.

Sonuç olarak, *pmrC*, *pmrA* ve *pmrB* deki ekspresyon artışı ile birlikte *pmrA, pmrB, pmrC* ve *lpx* genlerinde meydana gelen mutasyonlar kolistin direnci gelişimine katkı sağlamaktadır. En yüksek *pmrCAB* ekspresyonlarının maruziyetin üçüncü haftasında ve sonrasında görülmesi ile kolistin-dirençli örneğin klinikte kolistin tedavinin 25. günü izole edilmesi bize *A.baumannii*'de kolistin direnci gelişimi için uzun dönem tedavi gerekliliğini gösterdi. Kolistin ile tedavi süresi ve kolistin direncinde önemli faktörler olarak bulundu. Bunlara göre, kolistin tedavisi sırasında kolistin kullanım süresi ve kombine tedavi seçenekleri göz önüne alınmalıdır.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

cDNA	Complementary DNA
CLSI	Clinical and Laboratory Standards Institute
HAI	Hospital-acquired Infections
IAF	Intra-abdominal Fluid
ICU	Intensive Care Units
IMP	Imipenem
L-Ara4N	4-amino-4-deoxy-L-arabinose
LPS	Lipopolysaccharide
MDR	Multiple Drug Resistance
MH	Mueller Hinton
MIC	Minimum Inhibitory Concentration
NDM	New Delhi metallo-β-lactamase
OXA	Oxacillinase
PCR	Polymerase Chain Reaction
pEtn	Phosphoethanolamine
qRT -PCR	Quantitative Real Time PCR
SIM	Seoul Imipenemase
SNP	Single Nucleotide Polymorphism
TCS	Two-Component Regulatory System
TE buffer	Tris-Edta Buffer
TSA	Tryptic Soy Agar
VIM	Verona integron-encoded metallo-β-lactamase

1. INTRODUCTION

Hospital-acquired infections (HAI) caused by multidrug resistant (MDR) Gram negative pathogens became concerning worldwide. In Turkey, the most common causes of HAI are *Klebsiella pneumoniae (K.pneumoniae), Acinetobacter baumannii (A.baumannii), Escherichia coli (E.coli)* and *Pseudomonas aeruginosa (P.aeruginosa)* [1]. A recent study reported distributions of common Gram negative bacteria among 1156 patients diagnosed with gram negative bacteremia were as: 58% *A.baumannii*, 45% *P.aeruginosa*, 41% *K.pneumoniae*, and 28% *E.coli* with fatality rate of 42.4% [2]. Colistin is used as a last resort in antimicrobial therapy for MDR infections. However, colistin resistance is spreading among gram negative bacteria and makes treatment much more difficult. Colistin resistant *A.baumannii* isolates were obtained from different parts of the world such as Asia, Europe, and North and South America. Among 2217 clinical *A.baumannii* isolates from 17 different European countries, 5% of the total were resistant to polymyxins, and 80% of those polymyxin resistant isolates were obtained from Italy and Greece [3]. The situation in Turkey is also concerning. Ergönül and colleagues conducted a multicenter study in 2016 and reported colistin resistance rate 6%, but this rate was found to be 2% in 2018 [2, 4]. Therefore, an in-depth analysis of colistin resistance in *A.baumannii* is essential.

A.baumannii, a gram negative bacteria, is one of the ESKAPE pathogens and related to infections especially in immunocompromised patients with a prolonged hospital stay. Many publications investigated molecular mechanisms of colistin resistance in *A.baumannii*, but clinical factors are still unclear. In this thesis project, clinical resistance development by colistin use was mimicked in laboratory conditions to understand the relationship between host factors and molecular resistance mechanisms.

1.1 *Acinetobacter baumannii*

1.1.1 Characteristics, Structure and Identification of *A.baumannii*

A.baumannii is commonly found in soil and water., and belongs to Acinetobacter genus [5-7]. In Table 1.1, classification of *A.baumannii* is shown. It is a Gram negative, nonmotile and nonfermentative coccobacillus. Colonies are 1-2 mm, nonpigmented and mucoid. Different from Enterobacteriaceae, they cannot do nitrate reduction or to grow anaerobically [8, 9]. Colonies and image of Gram stained *A.baumannii* are shown in Figure 1.1.

Table 1.1: Classification of *Acinetobacter baumannii*

Domain	Bacteria
Kingdom	Eubacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
Family	Moraxellaceae
Genus	*Acinetobacter*
Species	*Acinetobacter baumannii*

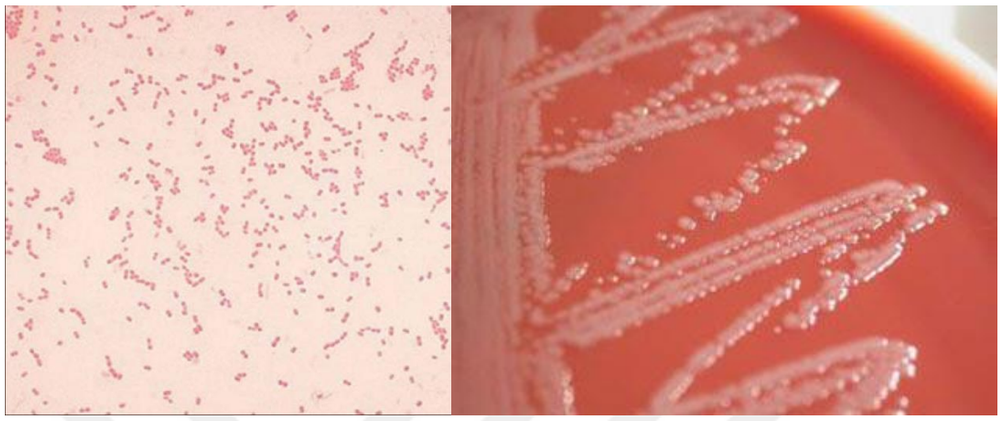


Figure 1.1: On the left, light microscopy image of Gram staining of *A.baumannii* can be seen. On the right, colony morphology of *A.baumannii* is shown.

A.baumannii is commonly detected in health-care facilities, especially in intensive care units. Being resistant to disinfection, to desiccation and to antimicrobials provides *A.baumannii* being a persistent pathogen in hospitals [10]. Biofilm formation ability is also reported to be very high among clinical isolates, which leads to transmission of infections with devices. *A.baumannii* can cause very severe urinary tract infections, wound infections, meningitis, bacteremia, and pneumonia. The transmission of infections caused by antibiotic resistant *A.baumannii* is much more concerning [8, 11, 12].

Classical phenotypic tests alone cannot identify *A.baumannii*. Epidemiological typing methods are used to distinguish the outbreak strain at subspecies level. These molecular typing systems are plasmid profiling, ribotyping, PFGE, randomly amplified polymorphic DNA analysis, rep-PCR, AFLP analysis, a high-resolution genomic fingerprinting method, integrase gene PCR, infrequent-restriction-site PCR and most recently, MLST and PCR-ESI-MS. *blaoxa-51* -like carbapenemase gene which is intrinsic to *A.baumannii* [13], or the *gyrb* gene in *A.baumannii* can be detected by PCR via specific primers. Similarly, MLST (multilocus sequence typing) scheme for *A.baumannii* was described as 305- to 513-bp sequences of the

conserved regions of the following seven housekeeping genes: *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* [10, 14, 15].

1.1.2 History of Antibiotic Resistance in *A.baumannii*

A.baumannii began to be a pathogen of hospital-acquired infections in the 1960s, when such infections could be easily treated with β-lactam antibiotics, because strains were generally susceptible to known antibiotics [6, 16]. Starting from 1975, resistant *A.baumannii* strains to β-lactams and sulfonamides emerged. Resistance mechanisms were mutations in penicillin-binding proteins, β-lactamase enzymes production, and alterations in membrane permeability. After 1975, imipenem, a carbapenem antibiotic, was used in infections caused by β-lactam and sulfonamide-resistant strains [17-19]. At the end of the1990s, treatment of *A.baumannii* infections became limited because of imipenem-resistant strains. During these years, carbapenem resistance dispersed quickly, with clonal spreading worldwide [20]. Carbapenem resistance mechanisms among bacteria mainly classified as alteration in drug target sites, enzymatic inactivations and active efflux or decreased influx of antibiotics. Carbapenem-hydrolyzing β-lactamases are common in carbapenem-resistant *A.baumannii*. VIM-, IMP-, SIM-type and NDM-1 metallo-β-lactamases were detected in *A.baumannii* carbapenem-resistant isolates, then class D oxacillinase gene clusters have been identified in *A. baumannii*, represented by the *bla*$_{\text{OXA-23}}$-, *bla*$_{\text{OXA-24/40}}$-, *bla*$_{\text{OXA-58}}$-like genes, and *bla*$_{\text{OXA-143}}$ gene. Oxacillinases can be in chromosome or in plasmids, which makes possible its acquisition by horizontal gene transfer. Some insertion sequence elements; such as IS*Aba1*, IS*Aba2*, IS*Aba3*, or IS*18,* regulate acquisition and expression of oxacillinases [13]. In addition, plasmid-mediated carbapenamases contributed to the dissemination of carbapenem-resistant *A.baumannii* [21].

Currently, colistin is used as a last resort for treatment of MDR infections, due to spreading resistance to many antibiotic classes. During colistin treatment of patients with MDR *A.baumannii* infections (including pneumonia, bacteremia, sepsis, intra-abdominal and central nervous system infections), 57-77% rate of cure or improvement was reported [22]. However, colistin-resistant *A.baumannii* is also spreading in hospital-acquired infections, with increasing usage of colistin. In 2001, a colistin-resistant *A.baumannii* was isolated from a patient who received colistin in order to cure an MDR *A.baumannii* infection [23]. Colistin resistance rates among *A.baumannii* were found in Spain (40%) and Bulgaria (16.7%) [24]. According to a study from Greece, between 2012 and 2014 all colistin-resistant *A.baumannii* isolates from a hospital belong to 3LST ST101 clone, and since 2011 all colistin-susceptible carbapenem-resistant isolates have been identified as ST101, too. This clone might became resistant under colistin stress factor and spread among this hospital [25]. In 2016, Ergönül et.al. reported colistin resistance rate as 6% among *A.baumannii* strains isolated from 17 ICU in Turkey [4]; however, surprisingly *A.baumannii* colistin resistance rate among 20 tertiary care centers in Turkey decreased to 2.1% in comparison with previous study [2].

Another important problem among *A.baumannii* is heteroresistance. Heteroresistant strains are resistant to certain antibiotics *in vivo*, but they are detected as susceptible *in vitro*. Common susceptibility tests cannot detect heteroresistance. This situation leads to improper treatments, and colistin-resistant *A.baumannii* rates can be more than estimated [26, 27].

1.2 Colistin and Colistin Resistance Mechanisms in *A.baumannii*

In order to fight against increasing mortality caused by colistin resistance in *A.baumannii*, it is important to understand mechanisms contributing the development of resistance.

1.2.1 Colistin

Colistin, also known as polymyxin E, is a polymyxin antibiotic, and belongs to a group of cyclic polypeptides [28]. Polymyxin is an old antibiotic discovered in 1947 from *Paenibacillus polymyxia*. Polymyxin B and colistin were synthesized by *P.polymyxa* as secondary metabolite nonribosomal peptides [29]. Japan and Europe used colistin therapeutically in 1950s and in USA, it is used in 1959 as colistimethate sodium. Colistin (polymyxin E) and polymyxin B are only clinically-used polymyxins. However, they have not been used for years because of their high nephrotoxicity and neurotoxicity [30, 31]. Nephrotoxicity occurs because of increase permeability in tubular epithelial cell membrane. This increment leads to cation, anion and water influx, eventually cell swelling and cell lysis. Colistin nephrotoxicity is affected by some risk factors, such as dose and duration of colistin therapy, coadministration of other nephrotoxic drugs, and patient-related factors [32]. Clinical use of colistin came to the fore again with the dissemination of MDR hospital-acquired infections. Decline in antimicrobial drug development also causes colistin to be the last choice in therapy [33].

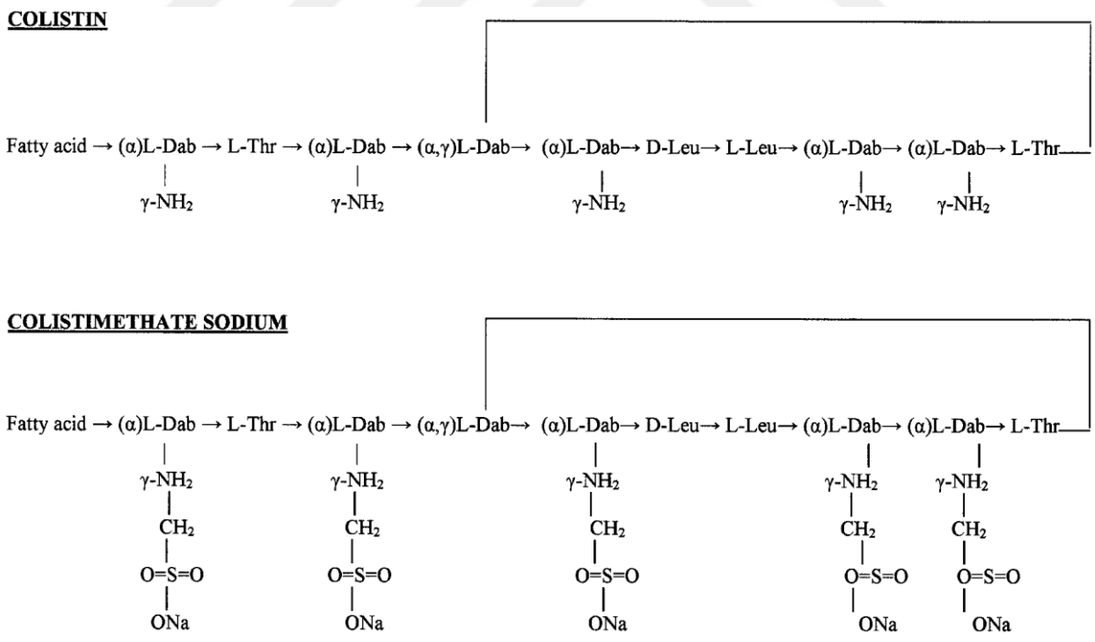


Figure 1.2: Chemical structure of colistin and colistimethate sodium. The fatty acid molecule is 6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B.

The structure of colistin is described in the Figure 1.2. Colistin is a multicomponent polypeptide antibiotic. Polymyxin B and colistin have similer primary sequence except replacement of D-Phe in polymyxin B to D-Leu in colistin at position 6 [29]. Two commercially available forms of colistin are colistin sulfate and colistimethate sodium. If colistin reacts with formaldehyde and sodium bisulfate, a sulphomethyl group is added to the primary amines of colistin and colistimethate sodium forms. Colistimethate sodium is less potent and also less toxic than colistin sulfate [29, 30]. Colistin has a strong positive charge in its polycationic peptide ring. The target of colistin is LPS molecules located on bacterial outer membrane. Colistin acts by replacing divalent cations from outer face of bacteria, so it causes disruption of cell and eventually cell death [33]. Some studies argued that polymyxin antibiotics may have multiple cell targets. Some studies showed that accumulation of reactive oxygen species in the cell (especially in Acinetobacter) is another suggested mechanism [34, 35]. Among these mechanisms, binding of colistin to lipid A portion of LPS causes blocking of endotoxin activity of microorganism in the host [30]. The main acting mechanism of colistin is illustrated in Figure 1.3.

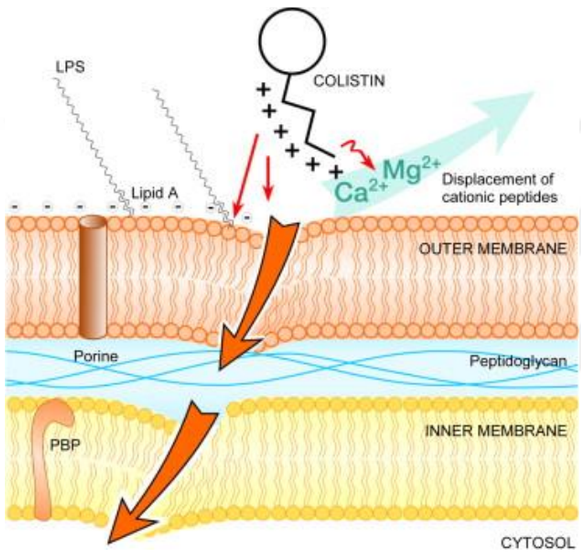


Figure 1.3: Colistin acting mechanism

1.2.2 Colistin Resistance Mechanisms in *A.baumannii*

In gram negative bacteria, mainly lipid A moiety of LPS forms shell structure of outer membrane. Lipid A shows endotoxic activity and is recognized by mammalian innate immune system receptors. In *A.baumannii*, two main mechanisms provide resistance to colistin: modifications in lipid A structure and complete loss of LPS [36].

Lipid A biosynthesis pathway consists constitutive and variable parts. Constitutive Lipid A biosynthesis enzymes and their single-copy genes are conserved among gram negatives except organisms like *Sphingomonas*, which produce sphingolipids instead of lipid A. *Lpx* genes (especially *lpxA*, *lpxC* and *lpxD*) play leading role in constitutive enzymatic pathway of lipid A biosynthesis, and they are generally not a target of regulation. However, complete loss of

LPS due to mutations or insertions in *lpxA*, *lpxC* and *lpxD* genes confer colistin resistance in *A.baumannii*. Moffatt and colleagues detected mutations in *lpxA*, *lpxC* and *lpxD* genes in independently derived colistin-resistant variations of standart strain (ATCC 19606) and in a colistin-resistant clinical isolate. Also, an 873-bp insertion sequence (IS) element, causing the inactivation of *lpxD* gene, is identified in the clinical isolate [37]. In later studies, they identified insertion of ISAba11 in *lpxA* and *lpxC* genes, resulting in the inhibition of LPS production and elevated MIC values of colistin [38]. Hua and colleagues obtained a colistin-resistant strain by *in vitro* colistin exposure, and whole genome analysis revealed that colistin resistance mechanism was LPS loss resulted from IS*Aba1* insertion in *lpxC*. In addition, occurrence of colistin resistance rapidly from MDR *A.baumannii* suggested that MDR *A.baumannii* has a high risk for emergence of resistant strains in clinical colistin use [36].

Although constitutive enzymes are intracellular components, modification enzymes are generally found either on periplasmic space of the inner membrane or in the outer membrane. Lipid A modification systems differ among gram negatives. The main mechanism is a cationic group addition to the lipid A part of LPS, which results in reducing net negative charge of bacterial outer membrane and reducing susceptibility to polymyxins and other cationic peptide [39, 40]. In gram negative bacteria, certain regulatory systems control the addition of positively-charged groups to LPS: PhoPQ and *PmrA*B two-component regulatory systems (TCS) [41, 42]. *A.baumannii* does not have PhoPQ system, but it is later discovered that *pmrCAB* (*pmrC* is phosphoethanolamine transferase) operon is responsible for addition pEtn in *A.baumannii*. Independent mutations and expression alterations in *PmrA/PmrB* TCS proved the significant role of *pmrCAB* operon in colistin resistance [43]. Upregulation of *pmrCAB* operon provides synthesis and addition of pEtn, which confers resistance to colistin [40]. Mutations that cause to *pmrCAB* upregulation are illustrated in Figure 1.4. Despite the majority of studies found that mutations in *pmrB* gene are mainly responsible to confer colistin resistance, Oikonomou and colleagues correlated colistin-resistant strains with mutations in *pmrA* and *pmrC* genes [25]. Activation of lipopolysaccharide-modifying genes involved in polymyxin resistance and subsequent pathways in Gram-negative bacteria is illustrated in Figure 1.5.

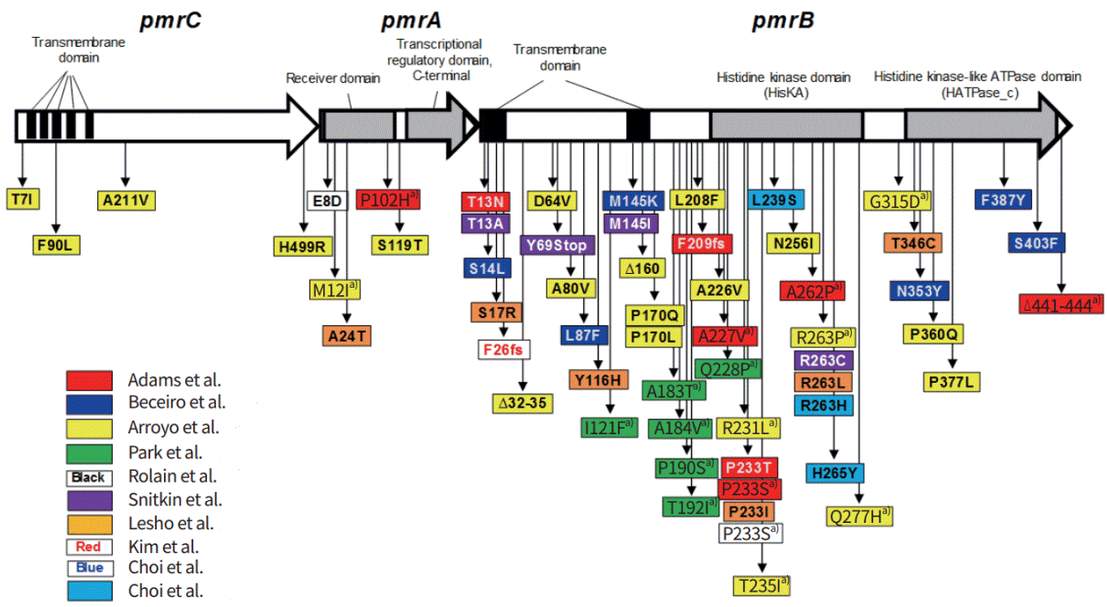


Figure 1.4: Overview of amino acid substitutions associated with colistin resistance in the polymyxin resistance (pmr) operon in *A.baumannii*.

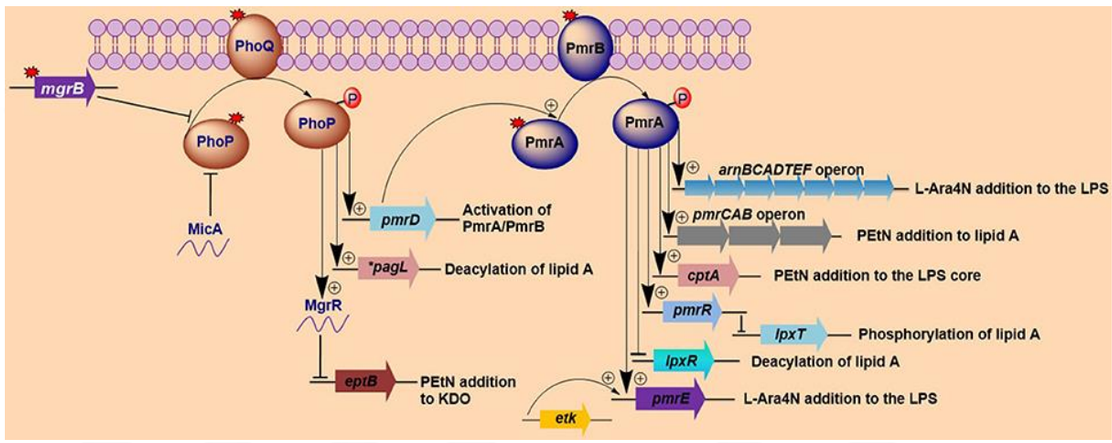


Figure 1.5: Activation of lipopolysaccharide-modifying genes involved in polymyxin resistance in Gram-negative bacteria

Studies also revealed additional mechanisms cause the colistin resistance in *A.baumannii*: NaxD deacetylation regulated by *pmrB* and insertion of two IS*15* in the *mutS* gene encoding DNA mismatch repair protein in colistin-resistant *A.baumannii* isolates [44, 45]. Other mechanisms were reported in other gram negative bacteria, like efflux pumps and capsule polysaccharide overproduction, but there are some contradictory data in literature. Choi and Ko reported reduced production of capsule polysaccharide in colistin-resistant *Klebsiella pneumoniae* [46]. A study about effect of an efflux inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), showed that CCCP increases colistin susceptibility, but other efflux inhibitors does not have such effect on *A.baumannii* [47]. Different *mcr* genes identified in colistin-resistant gram negatives prove the plasmid-mediated dissemination of colistin

resistance; however, as far as we know there are not any reports about the presence of *mcr* in colistin resistant *A.baumannii* isolates.

1.3 Fitness-Cost and Adaptive Evolution

The most important factor of colistin resistance was considered as exposure to colistin [48]. However, colistin resistance development can be seen in patients who were not under colistin treatment. This proves that there are another mechanisms and factors influencing resistance development to colistin [49]. Colistin resistance continues to increase despite advancing treatment techniques; therefore, investigating clinical factors and comparing them with *in vitro* experiences gain importance. In a case-control study conducted in 2008, risk factors were chosen as age, period in ICU, operations, colistin usage, monobactam usage, and the duration of colistin usage, and only risk factor contributing to colistin resistance was found as usage of colistin [50].

Gram negative bacteria can adapt to different stress conditions under exposure. They either use their own cellular machines or develop novel mechanisms to adapt stressful conditions. During adaptation process, genetic alterations occurring in their DNA provide them superiority, and allow to survive under exposure to stress like antibiotic presence. Genetic changes, such as single nucleotide polymorphisms (SNPs), insertions, deletions, transferring mobile elements and horizontal gene transfer play important roles in developing multiple drug resistance mechanisms. Bacteria modify their LPS structure to survive under colistin exposure. Antibiotic usage causes specific mutations in TCSs like *pmrA/pmrB,* which lead to expression changes in genes responsible for LPS modification [51-55]. Nhu and colleagues conducted a research in 2016 to understand adaptation mechanisms of *A.baumannii* to stress conditions. They obtained resistant isolates from susceptible ones under increasing colistin concentrations. Whole genome analysis revealed 6 mutations in *lpxACD* and 3 mutations in *pmrB*, where the most of which were novel [56].

Natural resistance mechanisms of bacteria to different antimicrobial agents can be considered as a subtype of virulence factors. Natural resistance mechanisms increase pathogenicity of bacteria and cause chronic diseases [57]. On the other hand, resistance mechanisms that bacteria developed against stress conditions have some costs for bacteria, which is called fitness-cost. A complex relationship between virulence factors and resistance mechanisms are present in this fitness-cost concept. Bacteria adjust this complex relationship via their genetic transcriptional factors [58, 59]. Then, environmental suitability determines the destiny of the resistance mutation [60]. In a research, Beceiro and colleagues compared growth rates of *A.baumannii lpx* mutants with ATCC strain to understand the connection between virulence and adaptation. Growth rates of colistin resistant *A.baumannii lpx* mutants decreased significantly. This decline suggested the cost, which *A.baumannii* mutants pay during adaptation to colistin presence [61].

2. METHODS

2.1 Patient Selection

A 35-years old female patient stayed in American Hospital (Istanbul) intensive care unit between February and March 2016 was followed up for infection. The clinical progress of the patient was recorded and several cultures from different body sites were obtained. The colistin susceptiple and colistin resistance *A.baumannii* isolates were stored for further studies.

2.2 Microbiological Studies

Four colistin susceptible *A.baumannii* from sputum and intra-abdominal fluid (IAF) were isolated from patient after 2 days of her admission. After 25 days of colistin use, one colistin resistant *A.baumannii* IAF isolate was determined. Totally four colistin susceptible and one colistin resistant *A.baumannii* isolates from this patient were included to the study. General scheme of laboratory part of the project was illustrated in Figure 2.1.

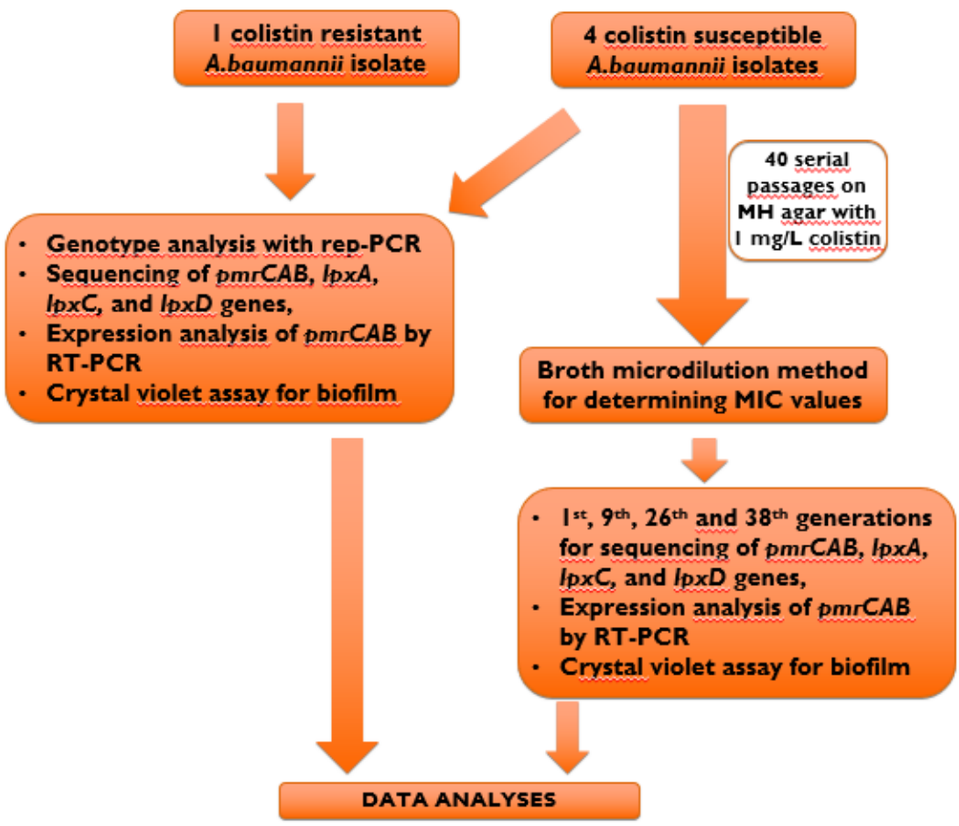


Figure 2.1: Laboratory phase of the study

.2.1 Serial Passages of Generations

Four colistin susceptible isolates were grown onto Mueller Hinton agar (MHA) (Becton, Dickinson and Company, U.S.) containing 1 µg/mL colistin concentration for total of 40 serial passages (each passage was named as generation). MIC values of all generations were assessed, and specific generations were chosen for molecular analysis.

2.2.2 Antibiotic Susceptibility Testing

Colistin minimum inhibitory concentrations (MICs) were determined by broth microdilution method in accordance with the Clinical Laboratory Standards Institute guidelines [62]. Isolates were grown on Tryptic Soy Agar (TSA) (Becton, Dickinson and Company, U.S.) overnight. 2 mL of cation-adjusted Mueller Hinton broth (MH) (Becton, Dickinson and Company, U.S.) with a McFarland 0.5 was prepared from each isolate. 100 µl of cation-adjusted MH broth was put into each well of a polystyrene round bottom 96-well plate. Cation-adjusted MH broth with 128 µg/mL colistin concentration was prepared in another Falcon tube. 100 µl of colistin – MH broth mixture was added by using multichannel pipette to first column of 96-well plate and serial dilution was performed by pipetting up and down except the last column, so colistin concentrations from 64 µg/mL to 0 µg/mL were obtained. 10 µl of samples was inoculated to each sample line and each sample was duplicated. Plate was incubated at 37°C overnight and MICs were determined by calculating of absorbance values at 540 nm. The resistance breakpoint was set as >2 µg/ mL according to the Clinical and Laboratory Standards Institute (CLSI) guideline. *E.coli* ATCC 25922 standard strain was used as reference control strain

2.3 Molecular Analysis

Total DNA and RNA isolation from clinical isolates and samples obtained in laboratory conditions was done in order to use for further molecular analysis. After overnight growth LB agar (Becton, Dickinson and Company, U.S.) at 37°C, DNA was extracted with the commercial DNA extraction kit according to the manufacturer's instructions (QIAGEN DNeasy UltraClean Microbial Kit, U.S.). Total RNA was extracted with the commercial RNA extraction kit (Macherey-Nagel, NucleoSpin RNA, Germany). Before RNA extraction, bacteria were grown overnight on LB agar (Becton, Dickinson and Company, U.S.) at 37°C then 5-10 colonies were inoculated into 5 mL LB broth and incubated in shaking incubator at 37°C, 125 rpm for 8 hours to obtain bacteria on logarithmic growth phase. After incubation, density was adjusted to McFarland 3 (~$9x10^8$) and cells were centrifuged at 4,500 rcf for 10 minutes to obtain cell pellet. Pellets were treated with lysozyme (1 mg/ml) (Sigma-Aldrich, Lysozyme from chicken egg white) which is diluted in TE buffer for lysing bacterial cells. RNA extraction protocol was followed according to the manufacturer's instructions. Concentrations of extracted RNA and DNA were determined by Thermo Scientific Nanodrop 2000 Spectrophotometer instrument. Extracted DNA was stored at -20°C, and RNA was kept at -80°C for molecular experiments.

Extracted DNA were further used in detection of *mcr-1* by PCR and genotyping of *A.baumannii* clinical isolates by rep-PCR based Diversilab system, and PCR and sequencing of *lpxA, lpxC, lpxD* genes and *pmrCAB* operon in all samples. Extracted RNA were used in qRT-PCR to detect *pmrA, pmrB* and *pmrC* expression levels after cDNA synthesis.

2.3.1 Genotyping

Clonal relatedness of the clinical isolates was determined by the repetitive PCR (rep-PCR) based diversilab system (Biomerux, France). For the repetitive PCR, DNA concentrations were adjusted to 25-50 ng/µl and rep-PCR mix was prepared as indicated in the Table 2.1. In the rep-PCR, primers specific for noncoding regions of bacterial chromosome was amplified with the Diversilab Acinetobacter Kit (Biomerux, France). In the PCR, AmpliTaq polymerase enzyme with GeneAmp 10x PCR buffer were used (Applied Biosystems, U.S.).

Table 2.1: Reagents used for rep-PCR

Reagent	**Volume/Reaction (µl)**
Rep-PCR MM1	18
GeneAmp 10X PCR Buffer	2,5
Primer Mix A	2
AmpliTaq DNA Polymerase	0.5
DNA (25-50 ng/µl)	2
Total Volume	**25**

PCR was carried out in Applied Biosystems Veriti 96 Well Thermal Cycler (Applied Biosystems, U.S.) with the following protocol: initial denaturation at 94°C for 2 min, 30 s of denaturation at 94°C (x35), 30 s of annealing 55°C (x35), and 90 s of extension at 70°C (x35), followed by 3 min of final extension at 70°C and ending at 4°C. After amplification, amplicons were loaded into DNA LabChip (Biomerux, France). After gel was loaded into the chip, 5 µl DNA marker, 1 µl ladder, and 2 µl of amplified products were loaded into assigned wells and separation was achieved with Agilent 2100 Bioanalyzer (Biomerux, France). The results were recorded and analyzed in DiversiLab Microbial Typing software system.

2.3.2 *lpxA, lpxC, lpxD* and *pmrCAB* PCR

For the amplification of 1179 bp *lpxA*, 1164 bp *lpxC*, 1502 bp *lpxD* and 3,699 bp *pmrCAB*, primers indicated in the Table 2.2 were used. Reactions were carried out with the commercial DreamTaq Green PCR master mix (Thermo Fisher Scientific, USA) which contains; 0.4 mM each of dATP, dCTP, dGTP and dTTP, and 4 mM $MgCl_2$. For the *lpxA*, *lpxC*, and *lpxD* PCR, following reaction conditions were used; initial denaturation at 95°C, 2 min., 35 cycles of denaturation at 95°C, 35 sec., annealing at 52, 55, 56°C, 35 sec., extension at 72°C, 45 sec. and lastly final extension at 72°C, 5 min. For the amplification of complete *pmrCAB* operon indicated conditions were followed; initial denaturation at 95°C, 2 min., 30 cycles of denaturation at 95°C, 30 sec., annealing at 58°C, 30 sec., extension at 72°C, 2 min. and lastly final extension at 72°C, 5 min. Amplicons were run on 2% agarose gel.

Table 2.2: PCR, qRT, and sequence primers used in this study

Oligonucleotide	Used for	Sequence	Source
lpxA-F	PCR/Sequence	TGAAGCATTAGCTCAAGTTT	Moffatt et. al. 2010
lpxA-R	PCR/Sequence	GTCAGCAAATCAATACAAGA	
lpxC-F	PCR/Sequence	TGAAGATGACGTTCCTGCAA	Moffatt et. al. 2010
lpxC-R	PCR/Sequence	TGGTGAAAATCAGGCAATGA	
lpxD-F	PCR/Sequence	CAAAGTATGAATACAACTTTTGAG	Moffatt et. al. 2010
lpxD-R	PCR/Sequence	GTCAATGGCACATCTGCTAAT	
Full *pmrCAB*-F	PCR	GCATCATAAAAAGATTGTAGTCAC	Beceiro et. al. 2011
Full *pmrCAB*-R	PCR	GCGATTTGTATTCATCGTTTTGAG	
pmrC-F	Sequence	ATGTTTAATCTCATTATAGCCA	Beceiro et. al. 2011
pmrC-R	Sequence	TTAGTTTACATGGGCACAA	
pmrC-F2	Sequence	GGTTGTTATTGAAGAAAGTAT	Beceiro et. al. 2011
pmrC-R2	Sequence	TCAATCCAAGTCACTTGGTAAC	
pmrB-F	Sequence	GTGCATTATTCATTAAAAAAAC	Beceiro et. al. 2011
pmrB-R	Sequence	TCACGCTCTTGTTTCATGTA	
pmrB-F2	Sequence	GGTTCGTGAAGCTTTCG	Beceiro et. al. 2011
pmrB-R2	Sequence	CCTAAATCGATTTCTTTTTG	
pmrA-F	Sequence	ATGACAAAAATCTTGATGATTGAAG AT	Beceiro et. al. 2011
pmrA-R	Sequence	TTATGATTGCCCCAAACGGTAG	
RT-*pmrA*-F	qRT	GGTGTTGCTGCTCTTTGACG	Adams et. al. 2009
RT-*pmrA*-R	qRT	GGTGGAATGGGTCAATAACG	
RT-*pmrB*-F	qRT	GAACAGCTGAGCACCCTTTAA	Beceiro et. al. 2011
RT-*pmrB*-R	qRT	ACAGGTGGAACCAGCAAATG	
RT-*pmrC*-F	qRT	CTCTTTACGCTTTGTTTTATGGAC	Beceiro et. al. 2011
RT-*pmrC*-R	qRT	GTAAAAAGTAAAACACCGACCA	
16S rRNA-F	qRT	TCAGCTCGTGTCGTGAGATG	Beceiro et. al. 2011
16S rRNA-R	qRT	CGTAAGGGCCATGATG	
CLR5-F	PCR	CGGTCAGTCCGTTTGTTC	Liu et. al. 2015
CLR5-R	PCR	CTTGGTCGGTCTGTA GGG	
OXA-23-F	PCR	GATCGGATTGGAGAACCAGA	Qi et. al. 2008
OXA-23-R	PCR	ATTCTGACCGCATTTCCAT	
OXA-24-F	PCR	GGTTAGTTGGCCCCCTTAAA	Qi et. al. 2008
OXA-24-R	PCR	AGTTGAGCGAAAAGGGGATT	
OXA-58-F	PCR	AAGTATTGGGGCTTGTGCTG	Qi et. al. 2008
OXA-58-R	PCR	CCCCTCTGCGCTCTACATAC	

2.3.3 Sanger Sequencing

Amplified products of *lpxA*, *lpxC*, *lpxD*, and *pmrCAB* were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Purified products were amplified with the 5 pmol of primers indicated in the Table 2.2 with the BigDye Terminator v3.1 Cycle (Applied Biosystems, U.S.) which amplifies products based on dideoxy-chain termination method. The indicated protocol was used to amplify products: 1 min. at 96°C followed by 25 cycles of 10 sec. at 96°C, 5 sec. at 50°C, and finally 4 min. at 60°C. The resulting products were purified with the ZR-96 DNA Sequencing Clean-up Kit (Zymo Research, U.S.) and sequences were detected with Applied Biosystems ABI 3500 Genetic Analyzer.

After sequence reads were obtained, ABI files were analyzed in Applied Maths Bionumerics version 7.5 Software (Biomerieux, France). Mutation analysis in *PmrA*, *PmrB*, *PmrC*, *LpxA*, *LpxC*, and *LpxD* proteins were done by aligning sequence reads and using ATCC 19606, ATCC 17978 and colistin susceptible clinical isolates K14&K178 as consensus sequence.

2.3.4 Carbapenamase Typing PCR

Carbapenamase typing was determined by the multiplex PCR that contains OXA-23, OXA-24 and OXA-58 primers (Table 2.2). These primers amplify 501 bp *bla*$_{\text{OXA-23}}$, 246 bp *bla*$_{\text{OXA-24}}$, and 599 bp *bla*$_{\text{OXA-58}}$. DreamTaq Green PCR master mix was utilized for PCR with the following conditions; initial denaturation at 94°C, 5 min., 30 cycles of denaturation at 94°C, 25 sec., annealing at 56°C, 40 sec., extension at 72°C, 50 sec. and final extension at 72°C, 6 min. Amplicons were separated on 2% agarose gel.

2.3.5 *mcr-1* PCR

To investigate the presence *mcr-1* gene, primers pointed out as CLR5 in Table 2.2 were used. PCR was carried out with DreamTaq Green PCR master mix and following conditions were used for amplification: initial denaturation at 94°C, 2 min., 35 cycles of denaturation at 94°C, 20 sec., annealing at 58°C, 30 sec., extension at 72°C, 30 sec. and final extension at 72°C, 5 min. PCR products were separated on 2% agarose gel.

2.3.6 *pmrCAB* Quantitative Real Time PCR

The reverse transcription of isolated total RNA was done with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) by using 500ng RNA. During the cDNA synthesis rDNAse was used to digest genomic DNA. Primers indicated in Table 2.2 were used for qRT PCR.

In the experimental procedure; 20µl reaction mixture for each sample in duplicate was prepared with 10 µl of LightCycler 480 SYBR Green I master mix (Roche, Germany), 3 µl of nuclease free water, 1 µl of 10 pmol from forward and reverse primers, and 5 µl of 1:10 diluted cDNA. The qRT PCR amplification was performed in LightCycler 480 II (Roche, Germany) with conditions indicated in Table 2.3.

Table 2.3: qRT PCR protocol for amplification of 16S rRNA, *pmrC*, *pmrB* and *pmrA*

Target (ºC)	Acqusition Mode	Hold (mm:ss)	Ramp Rate (ºC/s´)	Acqusition (per ºC)
Pre-incubation				
95	None	00:05:00	4.4	-
Amplification(x30)				
95	None	00:00:10	4.4	-
55	None	00:00:20	1.5	-
72	Single	00:00.30	4.4	-
Melting Curve				
95	None	00:00:05	4.4	-
65	None	00:01:00	2.2	-
97	Continuous	-	-	5-10
Cooling				
40	None	00:00:10	1.5	-

Relative gene expressions were calculated with the delta delta Ct method. 16S rRNA housekeeping gene was selected for normalization and *A.baumannii* standard strain ATCC 19606 was for calibration.

2.4 Crystal Violet Assay for Biofilm Assessment

Selected isolates were grown onto TSA plates at 37°C overnight. Single colony was inoculated into 5 ml TSB and incubated at 37°C until MacFarland reached ~10-13 (~16 hours/overnight). These cultures were diluted to 1:50 with TSB containing 1% glucose. 100 µl of diluted cultures were put into round bottom polystyrene 96-well plate (studied as triplicate). Plate was incubated in shaker at 37°C 100 rpm overnight. After this biofilm formation part, crystal violet assay was performed. Media in the wells of plate were discarded to remove medium with unattached bacteria. Then plate was washed two times with distilled water (with discarding the water at each step). 125 µl of 0.1% crystal violet was added to wells and plate was incubated for 15 minutes. Liquid in the wells of plate were discarded to remove unbound crystal violet and washed with distilled water. Plate was left to dry in incubator for 10 minutes. 200 µl of 95% ethanol was added to wells. Plate was incubated at room temperature for 15 minutes. OD values of wells were measured at 540 nm by Multiskan GO spectrophotometer.

3. RESULTS

3.1 Patient Follow-up

Two days after admission of the patient, 2 colistin susceptible A.baumanni isolates were identified from sputum and intra-abdominal fluid. Colistin therapy combined with meropenem and tigecycline was given for 25 days. After 25 day of colistin exposure, colistin resistant *A.baumannii* (MIC=16mg/L) was isolated from IAF. The clinical progression of the patient was shown in Figure 3.1.

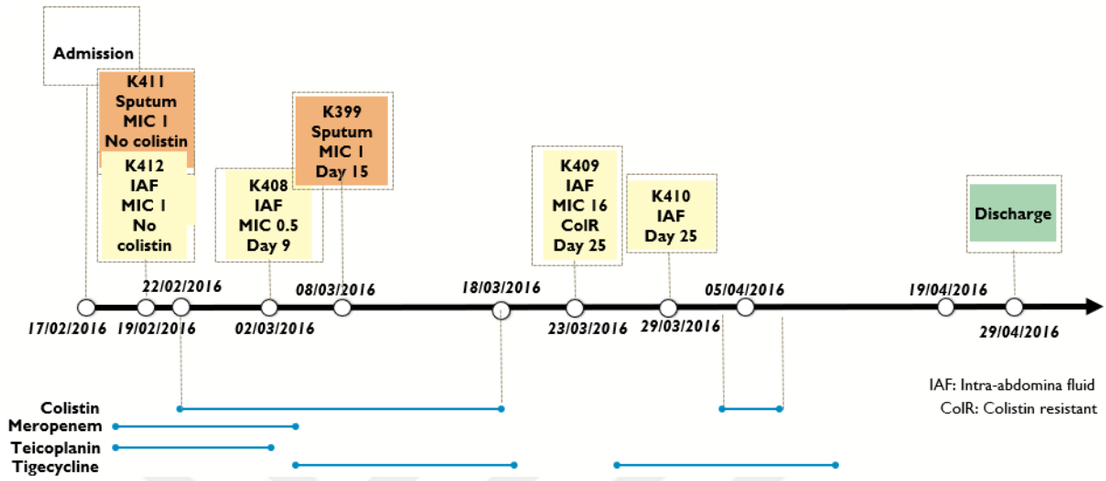


Figure 3.1: Clinical progress during hospitalization of the patient

3.2 Carbapenemase Type and *mcr-1* detection

Multiplex PCR which amplifies 501 bp *OXA-23*, 246 bp *OXA-24*, and 599 bp *OXA-58* revealed that all the *A.baumannii* clinical isolates carry *bla*$_{\text{OXA-23}}$. Figure 3.2 shows the PCR result of samples. For positive control, an OXA-23 positive carbapenem-resistant *A.baumannii* clinical isolate (K407) was used.

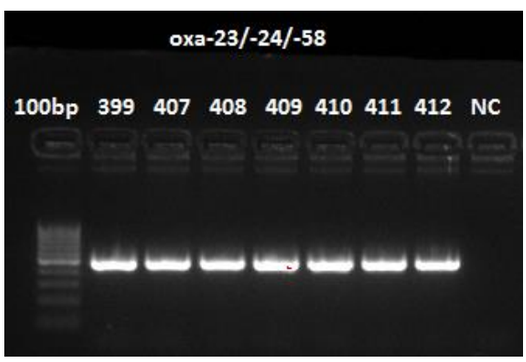


Figure 3.2: Agarose gel image of carbapenemase-typing multiplex-PCR which detects *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, and *bla*$_{\text{OXA-58}}$ of the samples

According to the PCR result which uses CLR5 primers to detect *mcr-1*, a plasmid mediated gene that encodes phosphoethanolamine transferase, none of the samples were carrying *mcr-1*.

3.3 Clonal Relatedness of Isolates

The dendrogram of one colistin-susceptible and colistin-resistant isolates isolated from the patient was shown in the Figure 3.3. Regarding to the similarity index, isolates which have more than 95% similarity are accepted as the member of the same clone. Accordingly, isolates belong to the same clone.

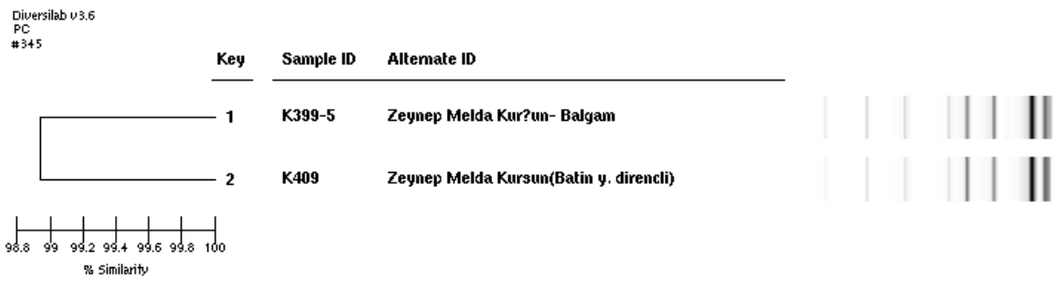


Figure 3.3: Dendrogram of one colistin-susceptible (K399) and colistin-resistant (K409) clinical isolates. Similarity index scale is on the bottom of the dendrogram. Rep-PCR gel images of samples were shown next to the dendrogram.

3.4 MIC Values

MIC values of isolates are shown in Table 3.1. MIC values of all generations determined by broth microdilution method can be seen in Table 3. 2.

Table 3.1: MIC values and sources of clinical *A.baumannii* samples

Isolation date	Sample code	Source	Colistin MIC values	Colistin resistance
19.02.2016	K411	Sputum	1	S
19.02.2016	K412	Intra-abdominal fluid	1	S
02.03.2016	K408	Intra-abdominal fluid	0.5	S
08.03.2016	K399	Sputum	1	S
23.03.2016	K409	Intra-abdominal fluid	16	R

Table 3.2: MIC values of generations during serial passages

	399	408	411	412
C0	1	0,5	1	1
C1	4	8	4	4
C3	4	8	4	8
C5	4	4	4	2
C7	4	8	2	4
C8	2	4	4	2
C9	4	8	4	4
C11	4	4	2	4
C13	2	4	2	4
C15	4	4	8	4
C17	2	4	4	2
C18	8	2	4	4
C19	2	2	4	2
C20	2	4	4	4
C21	2	4	4	2
C23	2	4	4	2
C24	4	4	8	2
C25	2	4	2	2
C26	8	8	4	8
C27	4	2	4	2
C30	4	2	4	2
C31	2	4	2	2
C32	2	2	2	2
C33	2	2	2	2
C34	4	4	4	2
C35	2	4	4	4
C36	4	4	4	2
C37	2	2	2	2
C38	4	4	8	4
C39	2	4	8	2
C40	8	4	8	4
C41	4	8	4	4

* Highlighted rows indicated chosen generations

**The lower MIC of duplicate study of each sample was presented

According to MIC results, 1st, 9th, 26th and 38th generations (indicated as C1, C9, C26 and C38) were chosen for further analyses. Colistin MIC values were above breakpoint after first passage. After 9th generation MICs were mostly stable above breakpoint. Generation 26 was selected because of being the timepoint of clinical resistance and generation 38 was chosen as endpoint of generations.

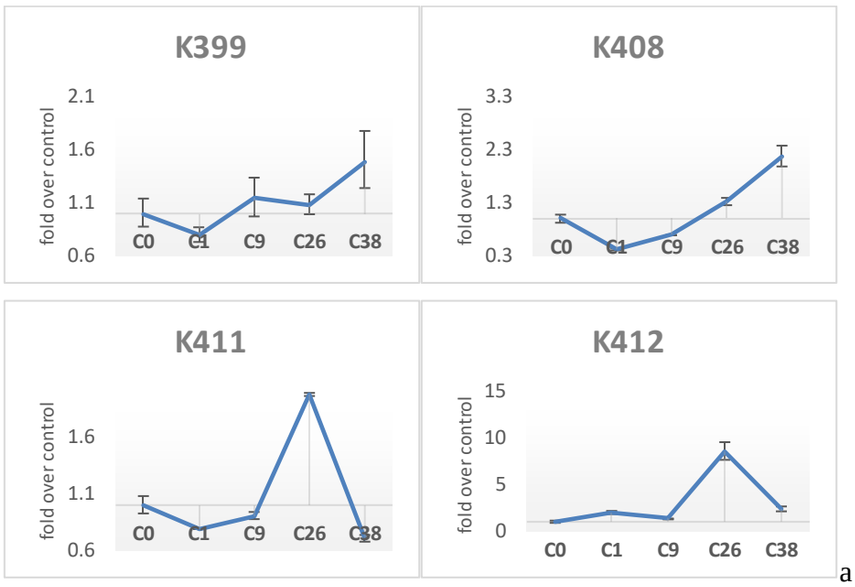
3.5 *PmrCAB* and *LpxA, LpxC, LpxD* Sequence Analyses

Multiple insertions were found in different regions of *pmrA, pmrB, pmrC, lpxA, lpxC,* and *lpxD* genes. No point mutations were detected.

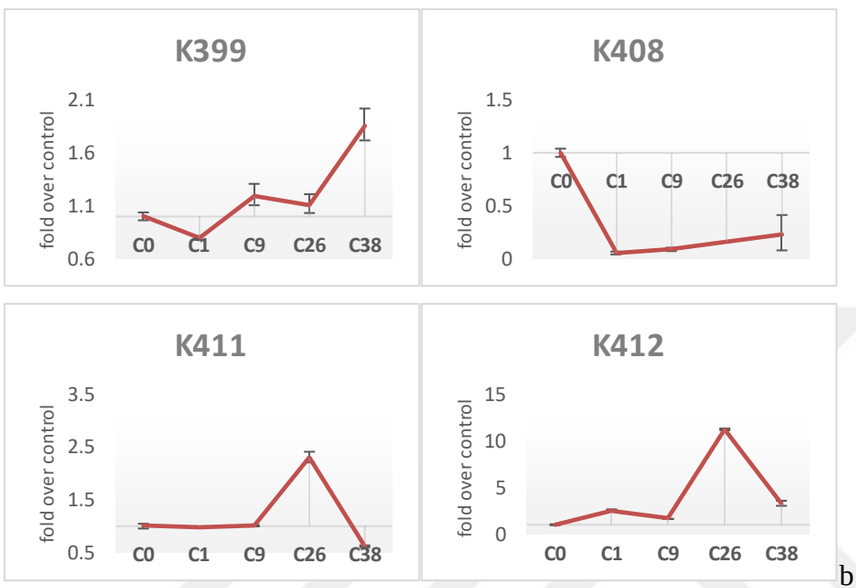
3.6 *pmrC, pmrA, pmrB* Expression Analyses

We compared *pmrCAB* expressions of colistin-susceptible (K412) and colistin-resistant (K409) isolates (both from intra-abdominal fluid) and found that *pmrA, pmrB* and *pmrC* genes were 1.6, 1.74 and 1.72-fold higher expressed in colistin-resistant isolate compared to susceptible one, respectively. Relative expression levels of *pmrC, pmrA* and *pmrB* genes of selected generations were indicated in Figure 3.4. According to qRT-PCR results, we saw different expression patterns between different isolates. However, generation 26 is the significant generation with the highest gene expression, especially for the isolates K411 and K412.

pmrA expressions decreased at first exposure to colistin except K412, then increased for all isolates. *pmrA* expression had its highest value at generation 26 for K411 (1.97-fold) and K412 (8.54-fold). *pmrB* gene was downregulated for K408. *pmrB* expression of K399 had the same pattern as its *pmrA* expression, but K411 and K412 upregulated at generation 26 (2.31 and 11.24-fold, respectively) and downregulated again at last generations. *pmrC* expressions of K399, K411 and K412 were at its highest value (2.11-fold, 4.03-fold and 29.65-fold, respectively) at generation 26 and then decreased at last generations, but *pmrC* of K408 upregulated till the last generation.



a



b

c

Figure 3.4: Relative expression levels of *pmrA* (Figure 3.4.a, indicated with blue), *pmrB* (Figure 3.4.b, indicated with red) and *pmrC* (Figure 3.4.c, indicated with green) genes of selected generations. *A.baumannii* ATCC 19606 was used as calibrator.

3.7 Crystal Violet Assay for Biofilm Assessment

Biofilm productivity of all generations were assessed by crystal violet assay and results were shown in Figure 3.5.

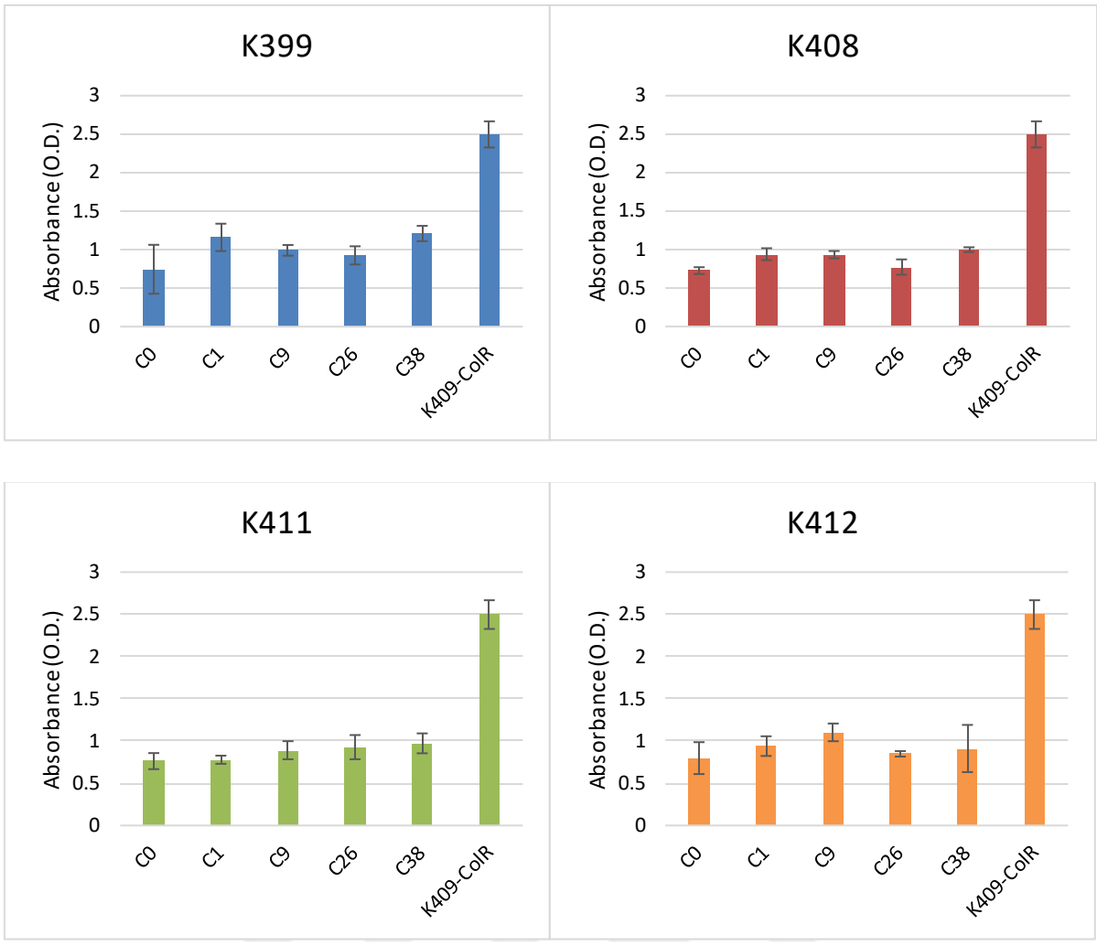


Figure 3.5: Crystal violet assay OD results measured at 540 nm for all generations. *Staphylococcus epidermidis* ATCC 35984 was used as control (mean absorbance O.D.: 0.8)

Crystal violet assay results show no significant difference between biofilm formation of generations and control group, but colistin-resistant clinical isolate has a stronger biofilm production capacity than the control group and all generations.

4. DISCUSSION

Colistin is used as the last option for therapy of MDR *A.baumannii* infections; however, dissemination of colistin resistance decreases success of treatment. Many researches showed development of colistin resistance either in laboratory environment or in clinical settings; however, the causes of resistance are still not well known. This study focuses on colistin resistance mechanisms in *A.baumannii* developed by colistin exposure both *in vivo* and *in vitro*.

Colistin exposure is found to be the main risk factor for development of colistin resistance by previous studies [48]. Matthaiou and colleagues reported that colistin use is the only risk factor for development of colistin resistance among a variety of risk factors [50]. Modifications in cell wall of *A.baumannii* prevents colistin binding and confers colistin resistance [39]. In our study, after 25 days of colistin treatment, colistin-resistant *A.baumannii* was isolated from intra-abdominal fluid of a patient. We compared *pmrCAB* expressions of colistin-susceptible (K412) and colistin-resistant (K409) isolates (both from intra-abdominal fluid) and found that *pmrA, pmrB* and *pmrC* genes were 1.6, 1.74 and 1.72-fold higher expressed in resistant isolate compared to susceptible one, respectively. The *pmrA/pmrB* TCS regulates the function of *pmrCAB* operon and the operon is responsible for addition of PEtn in cell membrane of *A.baumannii* [43]. Beceiro et al reported increased expression of *pmrA* (4- to 13-fold), *pmrB* (2- to 7-fold) and *pmrC* (1- to 3-fold) in resistant *A.baumannii* [63]. Upregulated *pmrCAB* genes in our clinical colistin-resistant isolate correspond to previous studies.

It is also known that point mutations in *pmrCAB* stimulates colistin resistance in *A.baumannii* [64]. In previous studies, point mutations in *pmrB* (a sensor kinase) were found to be responsible for colistin-resistant phenotype [43][56]. Our Sanger sequencing data of colistin resistant *A.baumannii* isolates revealed us presence of multiple insertions in *lpxA, lpxC, lpxD* and *pmrCAB*, but no point mutations. The reason of lack of point mutations in our resistant *A.baumannii* might be high clonal similarity of the isolates which was demonstrated in rep-PCR dendrogram. Whole genome sequencing could be more useful to reveal genetic mechanisms in-depth.

Many researchers studied evolutionary aspects of resistance in different bacterial populations. Çeliker and Gore worked with multispecies laboratory bacterial ecosystem to mimic the adaptive evolution process in nature and performed hundreds of generations by serial passages. [65]. Torres-Barcelo and colleagues studied effects of short and long-term evolutionary processes on SOS pathways in *P.aeruginosa* and performed daily passages with sublethal dose of ciprofloxacin presence [66]. Nhu and colleagues induced colistin resistance to different *A.baumannii* strains by exposing them to different concentrations of colistin according to their MIC values and daily passaged each survived strain to a medium with higher concentration of colistin [56]. Different experimental setups were used to investigate the effects of environmental stress factors on evolutionary processes in bacteria, like antibiotic

exposure. In our study, to mimic the effects of colistin exposure in colistin resistance development, we performed serial passages of colistin-susceptible isolates onto Mueller-Hinton agar containing colistin (1 mg/L) for 40 generations. Colistin MIC was above resistance breakpoint after first passage of *in vitro* experiments. We can postulate that colistin exposure has an effect on *A.baumannii*. Lee and colleagues investigated induced colistin resistance in *P.aeruginosa* and found that 6 days of 4 mg/L of colistin exposure was sufficient to gain colistin-resistant phenotype *in vitro* [67]. In a recent study, phenotypically colistin-resistant mutants of *A.baumannii* isolates were obtained after 24 hours of colistin exposure [68]. However, those colistin resistance induction studies emphasize the importance of heteroresistant phenotype of *A.baumannii* strains. The important part of our study is to contain clinically induced resistance data, as well. In our patient under colistin therapy, the resistance was detected at 26th day of colistin use. This can be explained additional contribution of host factors in clinical settings.

Development of colistin resistance is an adaptation to environmental stress [69]; therefore, it needs a time period for regulation of genetic and metabolic activities. It is shown that mutations and regulation of expression patterns are gained by stress-exposed bacteria in a certain period. Different bacteria and even different strains have diverse period to gain resistant-phenotype or virulence mechanisms. The expression studies of our evolutionary process showed that *pmrCAB* operon was expressed at different levels in each isolate in timeline of exposure. Surprisingly, *pmrCAB* expressions decreased at first generations of three out of four isolates. After having variable expression levels, the most significant finding of our study was the detection of highest expressions of *pmrCAB* being at 26th generation, which corresponds the isolation day of colistin-resistant *A.baumannii* in the patient. The most upregulated gene was *pmrC* in isolate K412 with 30-fold. We suggested that not only *pmrA*/*pmrB* TCS, but also *pmrC* (phosphoethanolamine transferase) is influential for resistance phenotype. Except *pmrB* downregulation in K408, other isolates showed upregulated *pmrCAB* operon genes especially at generation 26 and later (Figure 3.4).

Another interesting observation of our study was increased hypermucoviscosity phenotype of colonies at late generations (after 30). This finding could be because of increased capsule production, and for this reason we conducted biofilm experiments on our selected laboratory generations and clinical isolates. Biofilm formation is an important virulence factor and researches were conducted to reveal resistance-virulence and biofilm association in *A.baumannii*. In a study published in 2016, clinical colistin-resistant isolates showed deficient biofilm production compared to their susceptible counterparts [70]. Farshadzadeh and colleagues studied biofilm formation ability of clinical and laboratory-evolved colistin-resistant *A.baumannii* strains and found that mutations involved in colistin resistance phenotype might have negative affect on the expression of biofilm-associated genes of colistin-resistant isolates [71]. In our results, we did not find a difference in biofilm ability of our laboratory induced resistant generations. On the other hand, the biofilm production was three

times higher in the resistant clinical isolate than susceptible one. We suggest that clinical resistant isolate may developed other mechanisms to constitute colistin-resistance because of host-dependent factors plus environmental stress conditions and gained stronger biofilm capacity than laboratory-induced resistant isolates.

We conclude that, colistin exposure is the main factor for colistin resistance development. Colistin exposure leads to overexpression of *pmrCAB* operon, which contribute to colistin-resistance via LPS modification. Duration of exposure is important on *A.baumannii* for adaptation of colistin presence and establish colistin-resistant phenotype. After three weeks of colistin exposure, *A.baumannii* can develop a stable colistin-resistant phenotype with elevated MICs and upregulated *pmrCAB* operon both *in vivo* and *in vitro*. Stronger biofilm production in clinical resistance provides an additional support to virulence of *A.baumannii* in host.

5. BIBLIOGRAPHY

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