Host and Bacterial Factors Promoting Colistin Resistance in *Acinetobacter baumannii*

by

Elif Nurtop

A Thesis Submitted to the
Graduate School of Health Sciences
in Partial Fulfillment of the Requirements for
the Degree of

Master of Science
in
Medical Microbiology

Koç University

August 25, 2016

Koc University

Graduate School of Health Sciences

This is to certify that I have examined this copy of a master's thesis by

Elif Nurtop

and have found that it is complete and satisfactory in all respects,

and that any and all revisions required by the final

examining committee have been made.

Committee Members:

_____

Assoc. Prof. Füsun Can (Advisor)

_____

Prof. Dr. Önder Ergönül

_____

Prof. Dr. Kenan Midilli

Date: August, 25th 2016

ABSTRACT

Health-care associated infections (HAIs) have become a significant life-threatening problem over the time because of global increase in antibiotic resistant bacterial population. *Acinetobacter baumannii* (*A.baumannii*) is capable of survive in hospital environment for long time periods and cause severe infections with high mortality rates especially in intensive care units because of its multi-drug resistant (MDR) phenotype. Colistin is one of the last therapeutic options for treatment of MDR gram negative infections. Today, emergence of colistin resistance is a global concern because of the limitation of antimicrobial therapy. There are two mechanisms of colistin resistance in *A.baumannii*. Point mutations in *pmrCAB* operon induce PEtn addition on LPS which inhibits the colistin binding. Second mechanism is the prevention of colistin binding to cell wall by complete loss of LPS as a result of mutations or insertions in *lpxA, lpxC,* and *lpxD* genes. In this study, we aimed to reveal the association between the host demographic factors, clinical progression of the disease, and molecular variations of *A.baumannii* which may promote colistin resistance.

A total of 29 colistin resistant *A.baumannii* isolates from 4 different hospitals located in different parts of Turkey were collected between April 2015 and June 2016. Patient's demographic data, clinical progress of the disease, and outcome were recorded. To find the genetic basis of resistance, *pmrCAB* complete operon, *lpxA, lpxC,* and *lpxD* genes were sequenced. The expression levels of *pmrC*, *pmrA*, and *pmrB* were studied to find a connection between mutations and altered expression levels. The patient's data and the results of molecular tests were analyzed by using R Studio program.

In PmrA, PmrB, PmrC, and LpxD several amino acid changes were detected. The most common amino acid changes were T138A in PmrB, V42I& F150L in PmrC, and E117K in LpxD regions, and these mutations were found to be associated with the high colistin MIC values (p: 0.0022, 0.00086, 0.00086, 0.00086 respectively). According to the qRT-PCR results, 35.3-fold increase in *pmrC*, 12.3-fold in *pmrA*, and 8.2-fold in *pmrB* were detected. The duration of colistin therapy was found to be significantly associated with the overexpression of *pmrB* (p: 0.046). Having operation within one month before the isolation of colistin resistant *A.baumannii* is found to be significantly associated with overexpression of *pmrB* (p: 0.039).

In conclusion, the overexpression of *pmrC, pmrA,* and *pmrB* along with the point mutations in corresponding genes and *lpxD* gene may trigger colistin resistance. The duration of colistin therapy and operation within last month before the isolation of colistin resistant *A.baumannii* are substantial factors on emergence of colistin resistance. Therefore, physicians should consider the therapeutic options during colistin therapy by controlling the duration of usage and combining colistin with other antibiotics. Surveillance cultures before the surgery should be encouraged and protective precautions should be taken to prevent the dissemination of resistance.

ÖZETÇE

Sağlık bakımı ile ilişkili enfeksiyonlar dünya genelinde antibiyotik direncinin artması ile birlikte yaşamı tehdit eden bir problem haline gelmiştir. Sağlık bakımı ile ilişkili enfeksiyonların en önemli etkenlerinden biri olan *Acinetobacter baumanni* ise hastane ortamında uzun süre yaşayabilmekte ve ciddi enfeksiyonlara neden olarak özellikle yoğun bakım ünitelerinde yüksek ölüm oranlarının ortaya çıkmasına sebebiyet vermektedir. Kolistin ise çoklu antibiyotik direnci taşıyan gram negatif mikroorganizmaların neden olduğu enfeksiyonlara karşı son çare olarak kullanılmaktadır. Günümüzde kolistin direncinin ortaya çıkması, antimikrobiyal tedavinin sınırlanması ile sonuçlandığı için dünya genelinde ciddi bir endişe meydana getirmektedir. *A.baumannii*'de kolistin direncine neden olan 2 farklı mekanizma vardır. *pmrCAB* operonunda meydana gelen nokta mutasyonları LPS'e PEtn eklenmesini indükleyerek kolistinin bağlanmasını engellemektedir. İkinci mekanizma ise *lpxA, lpxC* ve *lpxD* genlerinde meydana gelen mutasyonlar LPS'i tamamen ortadan kaldırılarak, kolistinin hücre duvarına bağlanması engellenmesidir. Biz bu çalışmada konak faktörlerinin, klinik farklılıkların ve moleküler varyasyonların *A.baumannii*'de kolistin direnci gelişimindeki etkilerini ortaya çıkarmayı amaçladık.

Bu amaçla 2015 Nisan ve 2016 Haziran tarihleri arasında Türkiye'nin faklı bölgelerinde bulunan 4 faklı hastaneden toplam 29 kolistin dirençli *A.baumannii* izolatı toplandı. Hastaların demografik bilgileri, hastalığın klinik seyri ve sonucu kayıt altına alındı. Kolistin direncinin genetik nedenlerini ortaya çıkarmak amacıyla; *pmrCAB* operonu, *lpxA, lpxC* ve *lpxD* genleri sekanslandı. Mutasyonlar ve gen ekspresyonundaki değişiklikler arasında bağlantı kurmak amacıyla *pmrC, pmrA,* and *pmrB* genlerinin ekspresyonları tespit edildi. Hastanın klinik bilgileri ve moleküler testlerin sonuçları R Studio programı ile analizi yapıldı.

PmrA, PmrB, PmrC ve LpxD proteinlerinde amino asit değişimleri gözlemlendi. İzolatlar arasında en sık görülen amino asit varyasyonları; PmrB'de T138A, PmrC'de V42I& F150L ile LpxD'de E117K olarak belirlendi. qRT-PCR sonuçlarına göre *pmrC*'de ortalama 35.3 kat, *pmrA*'da 12.3 ve *pmrB*'de 8.2 kat artış gözlemlendi. Kolistin tedavi süresi ile *pmrB* geninin ekspresyonu arasında istatiksel olarak anlamlı bir bağlantı bulundu (p:0.046). Kolistin dirençli *A.baumnnii* izolasyonundan önceki 1 ay içinde operasyon geçirmiş olmak ve yüksek kolistin MIC değerleri ile istatiksel olarak anlamlı bulundu (p:0.039).

Sonuç olarak, *pmrC, pmrA* ve *pmrB* deki ekspresyon artışı ile birlikte *pmrA, pmrB, pmrC* ve *lpxD* genlerinde meydana gelen mutasyonlar kolistin direnci gelişimine katkı sağlamaktadır. Kolistin ile tedavi süresi ve kolistin dirençli *A.baumannii* izolasyonundan önceki 1 ay içinde operasyon geçirmek kolistin direncinde önemli faktörler olarak bulundu. Bu sonuç hekimlerin kolistin tedavisi sırasında farklı tedavi seçeneklerini, kolistin kullanım süresini ve kombine tedavileri gözden geçirmesi gerektiğini göstermektedir. Direncin

yayılımını engellemek için operasyon öncesinde sürveyans çalışmaları yapılarak koruyucu önlemler alınmalıdır.

ACKNOWLEDGEMENTS

First of all special thanks and gratitude goes to my advisor Assoc. Prof. Füsun Can, who shared all of her academic knowledge and life experiences which inspires me to walk on this path. I am also grateful for her limitless support and faith in me.

I also want to thank sincerely to Prof.Dr. Önder Ergönül for his great support, advice, and compelling stories about life that we all happy to hear. I am grateful for knowing him so that I had a chance to learn the importance of the human factor in science.

I would like to thank our Microbiology Laboratory Team, particularly Nazlı Ataç who made microbiology laboratory more than a physical space by creating so many unforgettable memories which I will always remember with a warm smile.

I also would like to thank Dr. Fulya Bayındır Bilman, Dr. Özlem Kurt Azap, Dr. Şirin Menekşe Yılmaz for their significant contribution to this study. Their participation made this research possible. I also want to thank Dr. Mehmet Gönen for his great contribution in statistical analysis.

My sincere thanks go to all members of the KUSOM family, especially friends in Z41B for the relaxing environment, advices and support. I also thank my friend, Kamil Kavak for his great support which inspires me all the time.

Finally special thanks go to my family for always believing me and supporting my decisions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AIDS	Acquired Immune Deficiency Syndrome
CAUTI	Catheter-associated Urinary Tract Irnfections
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CLABSI	Central Line-associated Bloodstream Infections
CLSI	Clinical and Laboratory Standards Institute
Dab	2,4-diaminobutyric acid
ESBL	Extended Spectrum Beta-lactamase
HAI	Hospital-associated Infections
ICU	Intensive Care Units
L-Ara4N	4-amino-4-deoxy-L-arabinose
LPS	Lipopolysaccharide
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
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
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SENIC	Study of the Efficacy on Nosocomial Infection Control
SSI	Surgical Site Infections
TCS	Two-Component Regulatory System
TE buffer	Tris-Edta Buffer
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar

UHESA	Turkish National Nosocomial Infections Surveillance Network
USD	United States Dolar
VAP	Ventilator-associated Pneumonia
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World Health Organization
XDR	Extensively Drug Resistant

CHAPTER 1

INTRODUCTION

Acinetobacter baumannii (*A.baumannii*) is a gram negative bacteria responsible from nosocomial infections which is one of the most considerable problem related with the medical treatment due to the high antibiotic resistance. In recent years, importance of *A.baumannii* nosocomial infections is increasing because of the colistin resistance[1]. In order to focus on this growing problem, in the following sections general information about nosocomial infections, *A.baumannii* that is one of the causes of these infections, and resistance to colistin is introduced.

1.1. Nosocomial Infections

Nosocomial also referred as healthcare-associated infections (HAI) are caused by medical treatment in a healthcare facility[2]. In order to classify an infection as a HAI, patient should show clinical evidences of contagion within 48 hours of admission to the healthcare facility, 3 days after discharge or 30 days of an operation, which is the evidence that responsible microorganism is associated with the healthcare[3].

HAIs are always a life-threatening public health concern however over time they became more problematic due to the increase in elderly population as a result of modern medicine, the AIDS epidemics, rise in invasive medical device usage, improper and over utilization of antimicrobials[4-5]. They became such a critical problem that in 2002, World Health Organization (WHO) announced HAIs as a priority[6].

Since HAIs are one of the prominent concerns in medical history, various infection control strategies have been initiated to minimize them[3]. The first nationwide project for nosocomial infection control is the CDC's Study of the Efficacy on Nosocomial Infection Control (SENIC) initiated in 1985, U.S. The data from the early SENIC reports pointed out that nosocomial infections can be reduced by 32% with accurate infection control programs[7].

The understanding of factors cause nosocomial infections is another vital step to eliminate epidemics. Because of this, epidemiological studies have been done to reveal factors affecting the occurrence and spreading of HAIs. The microbial factors responsible from HAIs are: bacteria, fungi and virus. Among these microorganisms, the most prevalent ones are defined as; *Streptococcus* spp., *Acinetobacter* spp., enterococci, *Pseudomonas aeruginosa*, coagulase-negative staphylococci, *Staphylococcus aureus*, *Bacillus cereus*, Legionella and Enterobacteriaceae family members like *Proteus mirablis*, *Klebsiella pneumonia*, *Escherichia coli*, and *Serratia marcescens*[8] .These pathogens are responsible from more than

80% of reported nosocomial infections which are: catheter-associated urinary tract infections (CAUTI),) (32%), surgical site infections (SSI) (22%), ventilator-associated pneumonia (VAP) (15%), and central line-associated bloodstream infections (CLABSI) (14%)[9]. Patient factors are also influencing development of HAIs. The notable ones are age, condition of immune system, underlying diseases (chronic diseases, cancer, immunodeficiency), and medical interventions (mechanical ventilation, indwelling catheters)[10-11]. Third factor is the environmental components of healthcare settings such as ventilation type and building structurture[11-12]. Final element is microbial resistance arising mainly as a consequence of widespread usage of antimicrobials[11].

Today HAIs are still an important concern despite of numerous infection control strategies. The first crucial problem is the high morbidity and mortality rate of HAIs all over the world. According to the data collected from 14 European countries, mortality rate of HAI is determined as 16.8% in 1992[13]. Another study conducted in 2009 with the participation of 75 countries, the mortality rate in intensive care units (ICU) is calculated as high as 33% [14]. In U.S. every year out of 2 million of HAIs, approximately 90,000 deaths were reported[15]. Besides high mortality and morbidity, another significant issue related with the HAIs is the treatment cost. According to the CDC's reports, in U.S., for 1992 cost of the annual HAIs are calculated as 4.5 billion dollars and 6.65 billion dollars in 2007 [16].

HAI ratios in Turkey are similar to other developing countries with a rate of 14.7% (per 1000 ICU days) which is high compared with the developed countries[17-18]. According to the data from Turkish National Nosocomial Infections Surveillance Network (UHESA) which started collecting data in 2006, *Staphylococcus aureus* is the most encountered gram positive pathogen with 53% methicillin resistance. As data maintained from UHESA in 2010, Extended Spectrum Beta-lactamase (ESBL) production of gram negative nosocomial pathogens was 45.8% in *K. pneumoniae* and 40% in and *E.coli* while carbapenem resistance was 31% in *P.aeruginosa* and 69% in *A. baumannii*[19]. Another epidemiological study conducted in 17 different hospitals located in different parts of Turkey determined that in gram negative microorganisms the prevalent antibiotic resistant microorganism is *A.baumannii* with 94% carbapenems, 94% fluoroquinolones, 97% third generation cephalosporins 73% aminoglycosides and 6% for colistin resistance [20]. In Turkey, cost of the antibiotic resistant *P.aeruginosa* treatment is estimated as 100,04 USD (daily cost per pathogen) and 92.47 USD for *A.baumannii* [21].

1.2. *Acinetobacter baumannii*

This section describes *A.baumannii*, one of the most predominant microorganism cause nosocomial infections. Characteristics, identification methods, and history of antibiotic resistance are presented.

1.2.1. Structure and Identification of *A.baumannii*

A.baumannii is the member of Acinetobacter genus which is Gram negative, nonfermentative, and nonmotile coccobacillus commonly found in soil and water [22-23-24]. Figure 1.1 displays the light microscope image of coccus shaped *A.baumannii*. *A.baumannii* is the most studied member of genus because of the high isolation rates in health care institutions[25]. This microorganism is generally known as a pathogen responsible for nosocomial infections, however a study states that community acquired *A.baumannii* infections are increasing[26].

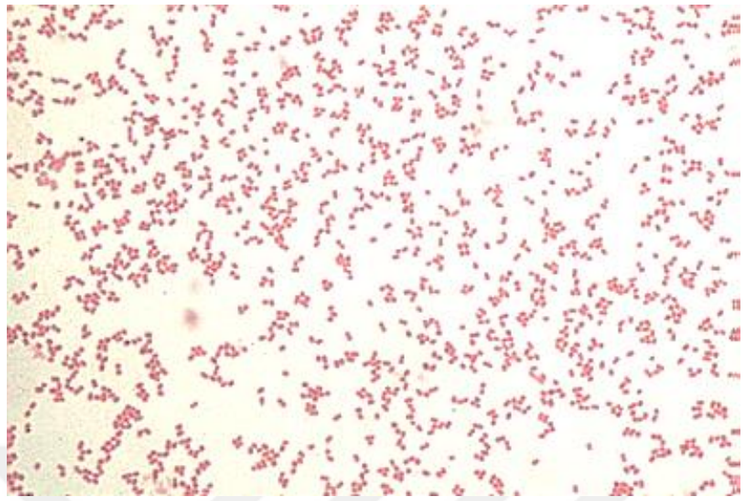

Figure 2.1 Gram stain of *A. baumannii* [27]

A.baumannii can grow under different temperatures, pH conditions, and with diverse energy sources which gives bacteria the ability to live on various surfaces for long time periods. This feature of *A.baumannii* partly explains the frequently encountered epidemics in health-care facilities particularly in intensive care units [27-28-29]. The biofilm formation ability is reported for more than 60% of the clinical isolates and this capability is the main cause of device-associated infections along with the increased resistance to certain antibiotics[30]. With the aid of all these properties, *A.baumannii* induces severe bacteremia, pneumonia, meningitis, urinary tract, and wound infections [31].

A.baumannii is one the common isolated microorganism in hospital setting but precise classification has preceded many years. In fact, many years after Beijerinck's first isolation of a microorganism in Acinetobacter genus which he named as *Micrococcus calcoateic*, Acinetobacter genus was not clearly classified[32]. In 1986 Bouvet and Grimont categorized 12 different groups of Acinetobacter by using DNA hybridization technique including *A.baumannii*[33]. Even today classical phenotypic tests used in microbiology laboratories alone cannot differentiate groups 1, 2, 3 and 13TU so that several research groups prefer to name these groups as *A. calcoaceticus-A. baumannii* complex[34-35]. However in recent years, molecular biological techniques advance detection of *blaoxa-51* like carbapenemase gene which is intrinsic to *A.baumannii*[36]. Another straightforward method is the PCR which is based on detection of *gyrb* gene in *A.baumannii* via specific primers[37].

1.2.2. History of Antibiotic Resistance in *A.baumannii*

In 1960s and 1970s, *A.baumannii* has been reported as nosocomial pathogen in Europe and U.S. In these reports almost all *A.baumannii* strains isolated from patients were susceptible to most of the known antibiotics and could be treated with β-lactams effortlessly [24-38]. Over the years, *A.baumannii* has acquired antibiotic resistance through various mechanisms, mainly through conjugation of plasmids[39]. Another mechanism explains the antimicrobial resistance is transposon transmissions which generally contains integrons. Similar to other gram-negative bacteria, different gene cassettes found in integrons give antimicrobial resistance to *A.baumannii* and eventually create Multiple Drug Resistant (MDR) phenotype[40]. These antimicrobial resistance genes give mainly three abilities: inactivation of antimicrobials by enzymes, blocking antimicrobial binding by making harder access to bacterial targets, and changing targets or cellular functions by mutations[41].

Starting from 1975, studies have revealed the ascending resistance in Acinetobacter clinical isolates[42-43]. Most of the isolated *A.baumannii* strains become resistant to older antibacterials like beta-lactams and sulfonamides by mutations in penicillin-binding proteins, production of beta-lactamase enzymes and change in the membrane permeability. After 1975, imipinem was chosen to treat beta-lactam and sulfonamide resistant isolates[44]. Another important problem in these years was the decline in manufacturing of new antibiotics[45].

During 1980s and 1990s big concern raised when imipenem resistance in *A.baumannii* was reported all over the world, because in these years imipenem was the first therapeutic option to eradicate resistant strains[46-27]. At the end of 1990s imipenem resistance was so spread that the therapeutic option for *A.baumannii* infections were became limited[41]. Later on as a result of fast clonal spread, carbapenem resistance began to disperse throughout the world[47]. Along with the clonal spread, plasmid mediated carbapenamase like OXA (Oxacillinase), NDM (New Delhi metallo-β-lactamase) and VIM (Verona integron-encoded metallo-β-lactamase) contributed to the dissemination of carbapenem resistant *A.baumannii*[48]. The carbapenem resistance distributions in 2014 are shown in Figure 1.2. In Turkey, Ergönül et. al., reported in 2013 that the carbapenem resistance in *A.baumannii* collected from the 17 different hospitals as high as 94% [20].

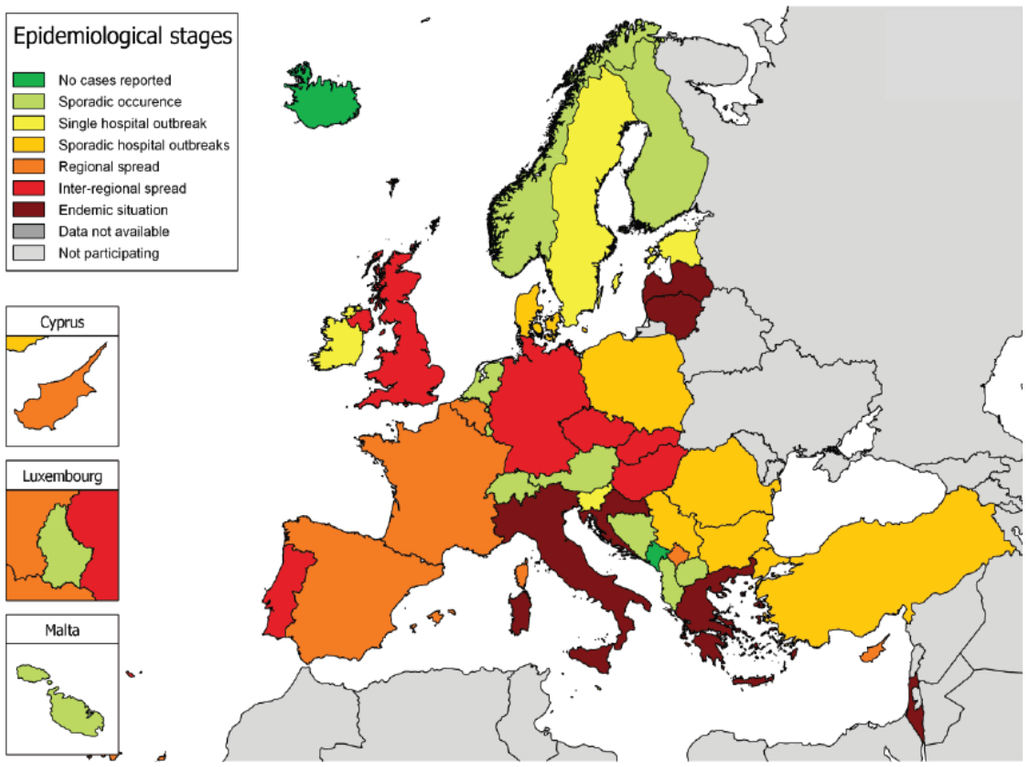

Figure 1.2 Carbapenem resistant *A.baumannii* distribution in Europe. The data collected from 38 countries in 2014 [49].

Since 2000 clinicians started to use colistin due to the wide spread of carbapenem resistant *A.baumannii*[50]. Unfortunately following from the colistin usage, colistin resistant *A.baumannii* strains began to be reported. One of the first colistin resistant *A.bauamnnii* was reported in 2001, isolated from a patient who received colistin in order to cure MDR *A.baumannii* infection [51]. In time, as a consequence of increasing colistin usage, the reports of resistance began to accelerate[52-53-54]. A study done in 2012, by observing studies done in Asia, Europe, and America predicted the general colistin resistance rate below 7%, however some countries show high resistance rates like 40% in Spain and 16.7% in Bulgaria[55]. In 2014, a study done with the participation of different healthcare centers located in Turkey estimated colistin resistance in *A.baumannii* as 5% [56].

Besides the rapid dissemination of Extensively Drug Resistant (XDR) *A.baumannii*, another problem is the reports of high heteroresistance. Heteroresistance is described by Falagas et. al. in 2008 as a subpopulation of microorganisms that are defined as susceptible to certain antibiotics by in vitro methods; although they are resistant in vivo. Since this situation cannot be detected by traditional susceptibility tests like minimum inhibitory concentration (MIC) used in diagnostic laboratories, heteroresistance causes increased treatment failure[57]. In a study, out of 16 *A.baumannii* clinical isolates, 15 were detected as heteroresistant to colistin which demonstrates that colistin resistant *A.baumannii* frequency may be more than it was anticipated with traditional methods[58].

Another crucial problem which contributes to the alarming colistin resistance is the excessive utilization of colistin to treat food animals. In 2012, it was predicted that the colistin was used to treat food animals 600 times more than to treat human[59]. The over utilization of colistin in veterinary is believed to contribute to the dissemination of plasmid born mcr-1 gene which generate colistin resistance in *Enterobacteriaceae.* In order to unravel this problem, especially in Europe governments began to ban and impose restriction to utilization of colistin in veterinary[60].

1.3. Colistin and Genetic Mechanisms of Resistance in *A.baumannii*

It is clear that understanding the molecular mechanisms of colistin resistance in *A.baumannii* is important to minimize the spread of this highly virulent and pathogenic microorganism. Therefore in this section, general information about colistin antibiotic and discovered molecular mechanisms of colistin resistance in *A.baumanni* is introduced.

1.3.1 Colistin

Colistin is a member of polymyxin antibiotics which is a peptide antibiotic isolated from *Bacillus polymyxa*. There are five types of polymyxins available on market but only polymyxin B and colistin (polymyxin E) have clinical usage [61].

Polymyxin B and colistin are similar in structure however only one amino acid differs between these peptides[62]. Molecular weights of the polymyxins are about 1200 Da. The structure of colistin and polymyxin B is described in the Figure 1.3. These polycationic peptide rings contain numerous 2,4-diaminobutyric acid (Dab) regions, and a fatty acid side chain linked with an amide bond [63]. The positively charged Dab regions found in polymyxins are important for creation of attraction between the negatively charged phosphate groups of LPS [64].

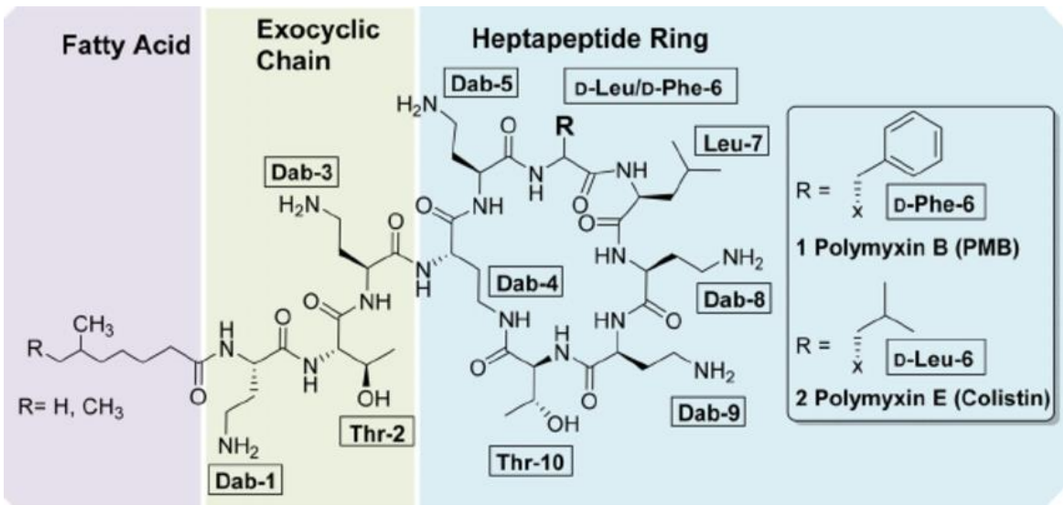

Figure 1.3 Structure of polymyxin E (colistin) and polymyxin B antibiotics [65]

These polycationic polymyxins show their affectivity against gram negative bacterial cell wall by disrupting the outer membrane which leads increase in permeability. Even many

years after discovery of polymyxins, exact action mechanism was not known. Later on it was revealed that polymyxins act by performing competitive divalent cation substitution with the Mg^{2+} or Ca^{2+} thus creating noncovalent bonding with the adjacent polysaccharide, which resulted with degradation of membranes and eventually cell death[66-67]. In Figure 1.4 the schematic representation of the action mechanisms of polymxins are illustrated. As the figure presents other than membrane lysis, additional mechanism named vesicle-vesicle contact also explains the osmotic imbalance in bacteria which leads lysis. According to this mechanism, polymyxins induce exchange between inner and outer membrane which is concluded with destruction of specificity between these leaflets and causes osmotic imbalance[68-69].

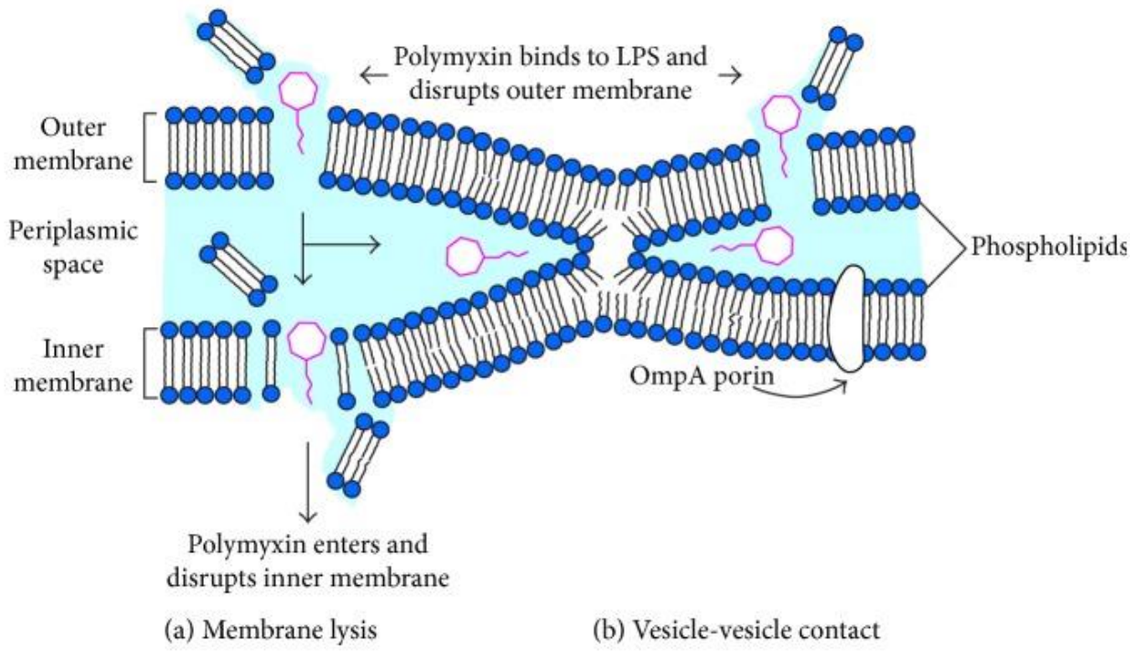

Figure 1.4 Mechanisms of polymyxin action on gram negative cell: (a) classical mechanism (b) alternative mechanism named vesicle-vesicle contact[70]

Last mechanism described more recently demonstrates the accumulation of reactive oxygen species (ROS) inside bacteria, induced by polymyxins. In *A.baumannii*, increase of $^{\cdot}OH$ production after polymyxin B or E treatment was established[71].

Since polymyxin-LPS binding has high affinity, polymyxins are defined as one of the most effective cell-permeating agents. Another advantage of using polymxins is that LPS wiped out from host's body thus the endotoxic effects released by LPS are abolished. This effect of polymyxins on LPS is clear in Scanning Electron Microscopy (SEM) images demonstrated in Figure 1.5.

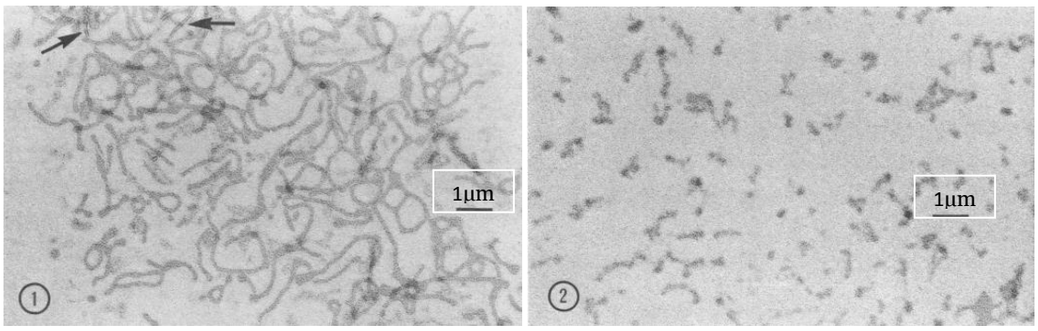

Figure 1.5 (1) SEM images of LPS from avian *E.coli*. Arrows indicate ribbon like structures that have densely packed in the outer part less than inner parts. (2) Cells after treatment with polymyxin[72].

Despite exceptional effects of polymyxins, they do not have a wide usage due to the nephrotoxic and neurotoxic effects. Because of these side effects, aminoglycosidase and betalactams have been chosen to treat infections for decades [63]. Polymyxins have only been used to treat cystic fibrosis patients persistent lung infections, ear and eye infections with topical usage until the isolation of colistin resistant strains [73].

1.3.2 Colistin Resistance Mechanisms in *A.baumannii*

After the first report of colistin resistant *A.baumannii* clinical isolate, two main resistance mechanisms were reported: structural modification of lipopolysaccharide(LPS) and complete loss of LPS[74-75].

In gram negative bacteria, the capsular polysaccharide and LPS have an essential function that they protect bacterial membrane from lysis. As shown in Figure 1.6, this important layer is formed from three different segments; lipid A known as the bioactive component, the core oligosaccharide, and O antigen[76].

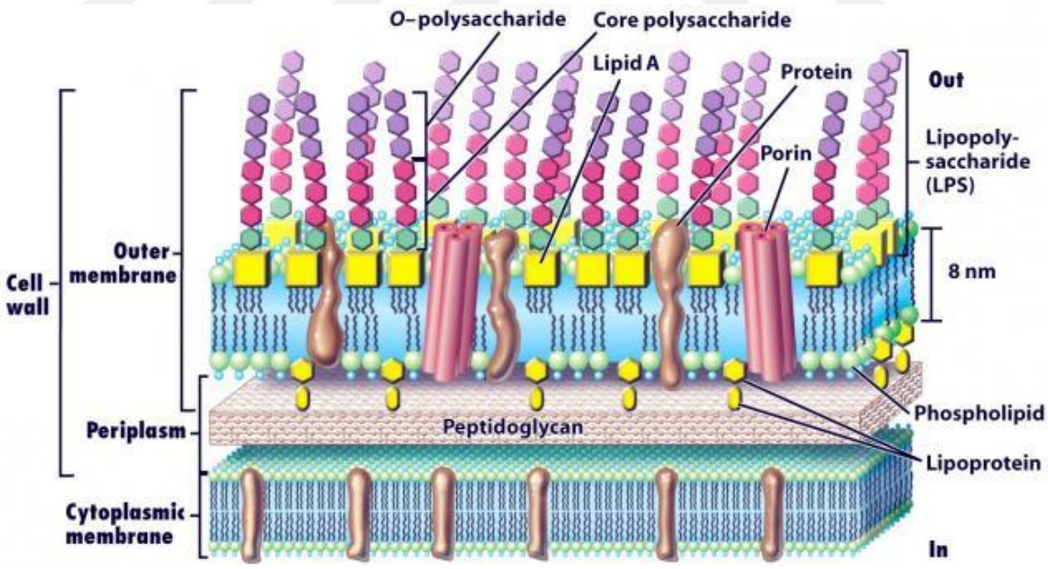

Figure 1.6 Structure of gram negative cell wall [77]

Before the discovery of PEtn addition in *A.baumannii*, it was shown that in *E.coli* and *Salmonella enterica* 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine

(PEtn) transferase expression is under control of PmrA/PmrB, a two-component regulatory system (TCS) [78]. This two component regulatory system is composed of a response regulator (PmrB) and sensor kinase (PmrA) and their expression levels are regulated by environmental variables like pH, Fe^{3+} and Mg^{2+} [79]. Although genes related with Ara4N synthesis and attachment were not be found, Adams and his coworkers demonstrated that pmrCAB (pmrC is phosphoethanolamine transferase) is also responsible for addition PEtn in *A.baumannii* which confers colistin resistance[74]. Later on several studies revealed that certain mutations in pmrCAB operon increase the PEtn addition on LPS which resulted with the reduction of colistin binding affinity to *A.baumannii*.

A study conducted with colistin resistant spontaneous mutant strains of ATCC 17978 revealed independent mutations in *pmrA* and *pmrB*, along with the increased *pmrA* expression. Reversion from colistin resistant to susceptible after partial deletion of *pmrB* supports that *pmrAB* TCS is significant for conferring colistin resistance in *A.baumannii* [74].

Another study demonstrates amino acid changes in PmrB occur as a result of *pmrB* mutation in a clinical isolate which caused nosocomial outbreak. This study also confirmed the increased expression levels of *pmrA* (4- to 13-fold), *pmrB* (2- to 7-fold), and *pmrC* (1- to 3-fold) in resistant strains compared with the susceptible strains[80]. Later on in several studies both conducted with colistin resistant clinical isolates and in vitro derived mutants, various mutations were detected in PmrC, PmrA, and PmrB which are summarized in Table 1.1

Table 1.1 Amino acid variations and changes related with colistin resistance in PmrCAB operon

	Amino acid variations	**Reference**
PmrC	T7I, F90L, A211V, H499R	Arrayo et al., 2011
PmrA	M12I, S119T, E8D	Arroyo et al., 2011
	E8D	Lesho et al., 2013, Rolain et al., 2013
PmrB	P102H , T13N, A227V, P233S, P233T, A262P	Adams et al., 2009
	I121F, A183T, A184V, P190S, T192I, Q228P	Park et al., 2011
	S14L, L87F, M145K, A227V, P233S, N353Y, F387Y, S403F	Beceiro et al., 2011
	I121F, A183T, A184V, P190S, T192I, Q228P	Park et. al., 20122
	ΔA32-E35, D64V, A80V, ΔL160, P170Q, P170L, L208F, A226V, R231L, P233S, T235I, N256I, R263P, R263C, Q277H, G315D, P377L	Arroyo et al., 2011
	S17R, Y116H, T232I, R263L	Lesho et al., 2013
	P170L, P233S	Pournaras et al., 2014
	A227V, P233S, FrF26	Kim et al., 2014b
	P233S, R263H	Lim et. al., 2015

Some studies demonstrated increase in expression level of *pmrC* in colistin resistant strains while others show no significant difference between colistin resistant and susceptible strains[81-82]. This situation is described by Lesho et al in 2013 with the detection of additional *pmrC*-like gene (*eptA*) that situated close to mobile elements[82].

The modifications on lipid A in colistin resistant *A.baumannii* were demonstrated with Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) distinctly. In order to show difference in LPS, Arrayo et. al. investigated the extracted lipid A in MALDI-TOF under negative-ion mode. The peaks of colistin susceptible standard strain ATCC 17978 and colistin resistant mutant 17978 R2 derived from wild type can be assessed in Figure 1.7. Another study done by using mass spectrometry indicated modified LPS with pEtN and galactosamine (GalN) in both colistin resistant laboratory-derived *A.baumannii* and clinical isolate[83].

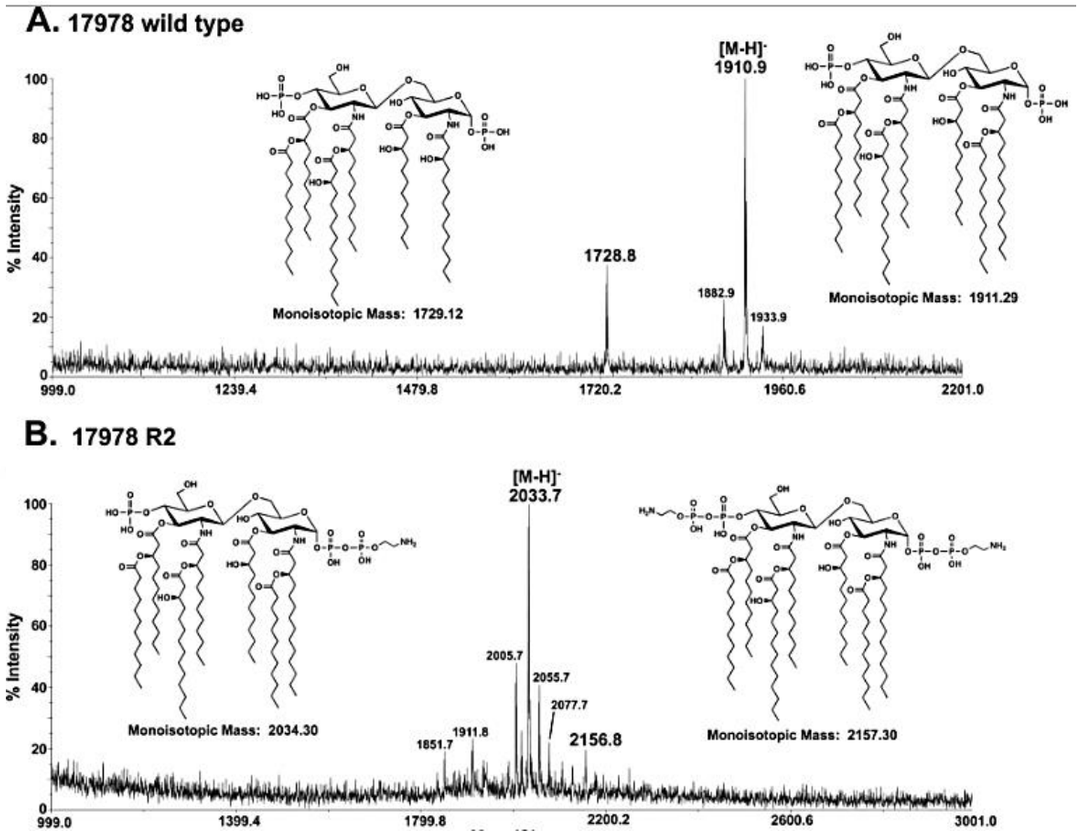

Figure 1.7 MALDI-TOF analysis of LPS isolated from *A.baumannii* (A) The m/z values of the wild type 17978 peaks were corresponding to bis-phosphorylated hepta-(1,910.9) and hexa-acylated (1,728.8) lipid A. (B) The m/z values of colistin resistant derivative were 2,033.7 and 2,156.8 which represents the bis-phosphorylated hepta-acylated lipid A contains one and two phophoethanolamine groups [81].

Second colistin resistance mechanism is the complete loss of LPS as a result of mutations or insertions in *lpxA, lpxC* and *lpxD* genes. These genes are in charge from lipid A biosynthesis pathway[84-75] . In 2010, Moffatt et. al. identified mutations in *lpxA, lpxC* and *lpxD* genes in independently derived colistin resistant variations of standart strain (ATCC 19606) as well as in a colistin resistant clinical isolate. The loss of LPS is also asserted by transmission electron microscopy (TEM) images designated in Figure 1.8 [75].

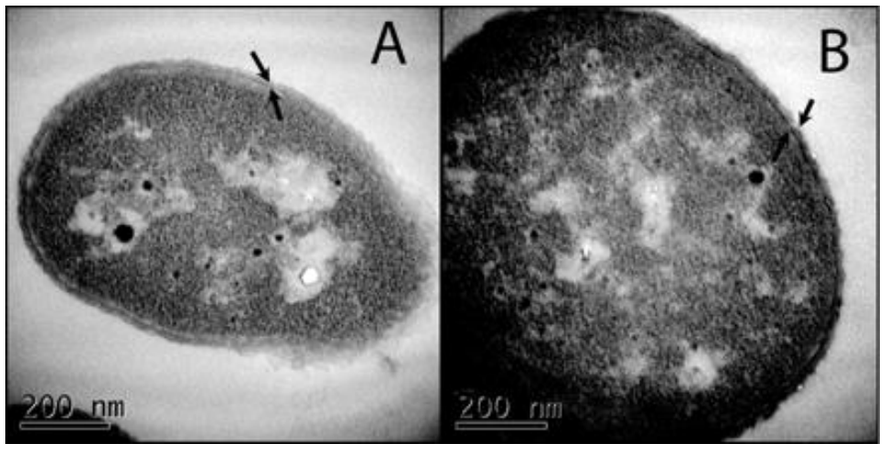

Figure 1.8 TEM images of *A.baumannii* **(A)**Arrows indicate the normal membrane of parent strain ATCC 19606. **(B)** Arrows show attenuated membrane of *lpxA* mutant of ATCC 19606

Other than the point mutations in *lpxA, lpxC* and *lpxD* genes, Moffatt et. al. first introduced the inactivation of *lpxD* gene of a clinical isolate from patient in South Korea by 873-bp insertion sequence (IS) element [75]. Later on by examining in vitro derived mutants of ATCC 19606, they identified insertion of ISAba11 in *lpxA* and *lpxC* genes which create the inhibition of LPS production and high colistin MICs[84].

It was shown that LPS deficient strains of colistin resistant *A.baumannii* overcome the lack of LPS by increasing expression of certain genes responsible from cell envelope and membrane biogenesis. For example Henry et. al. proved the raise in the expression of genes related with the synthesis and transport of the poly-β-1,6-N-acetlyglucosamine (PNAG), a surface polysaccharide in *lpxA* deficient colistin resistant mutants of *A.baumannii* when compared with the wild type strain [85].

In recent years several additional mechanisms related with the colistin resistance in *A.baumannii* were revealed. In 2015 Chin et. al. identified that NaxD deacetlyase, member of YdjC superfamily which is regulated by PmrB has a critical role in lipid modification. They established higher expression level (approximately 250 times higher) of *naxD* in *in vitro* derived colistin resistant mutant which has mutation in *pmrb*, compared with the wild type susceptible strain. Also with the aid of MALDI-TOF experiments, they showed GalN modification was affected in the *naxD* deleted mutant while no change occurs in pEtN addition to Lipid A [86]. In another study Lim et. al. identified insertion of 2 IS*15* in the *mutS* gene which encodes DNA mismatch repair protein, of a colistin resistant clinical isolate that can explain the high SNPs in colistin resistant *A.baumannii* [87].

Another colistin resistance mechanism lately described by Liu et. al., 2016 is the *mcr-1* gene which belongs to phosphoethanolamine transferase enzyme family. This mechanism particularly attracted notice because it is the first described plasmid-mediated polymyxin resistance mechanism, and pHNSHP45 plasmid that contains *mcr-1* is highly transferable [88]. Even though there is no *mcr-1* in *A.baumannii* reported, different studies all over the world have been presenting *mcr-1* in *E.coli*, *K.pneumoniae*, and *Salmonella Typhimurium* [88-89-90].

Another novel plasmid mediated mechanism of polymyxin resistance is identified in Belgium which is named *mcr-2*. On June, 2016 Xavier and his coworkers analyzed 10 colistin resistant *E.coli* isolated from porcine and bovine and found a novel phosphoethanolamine transferase (mcr-2) which shows 76.75% resemblance to *mcr-1* [91].

Along with the mechanisms of colistin resistance in *A.baumannii*, the effects of genetic changes in colistin resistant *A.baumannii* isolates is another important issue due to the understanding the clinical impacts. Beceiro *et.al*., 2013 demonstrated difference of fitness and virulence in colistin resistant isolates by comparing the ATCC 19606 with the laboratory derived *lpx* and *pmrB* mutants. They proved that all *lpxA*, *lpxC*, *lpxD*, and *pmrB* mutants showed *in vitro* fitness cost compared with the parent strain [92]. In 2013, Lopez-Rojas *et. al*., reported similar results in a study which compares the colistin susceptible *A.baumannii* clinical isolate with its consecutive colistin resistant strain. They showed that the growth rate of resistant strain is significantly lesser than the resistant strain. Similar to fitness, they also proved with the murine sepsis model that the virulence is also attenuated in resistant strain [93]. Interestingly, in mouse systemic-infection model experiment, among the laboratory derived mutants only *pmrb* mutant showed similar results to parent strain which proves that the loss of LPS has critical impact on virulence while addition of phosphoethanolamine to lipid A has no influence on virulence [92]. On the other hand a study done in 2015 did not identified altered fitness, growth rate, and virulence in a colistin resistant *A.baumannii* strain which has P233S mutation in PmrB. These findings demonstrate that along with the mutated gene type, the position of mutation may have an impact on fitness and virulence of the pathogen [94].

CHAPTER 2

METHODS

2.1. Bacterial strains

In this study 29 colistin resistant *Acinetobacter baumannii* isolates were included. Colistin resistant and susceptible strains were obtained from 4 different hospitals located in Turkey; Menemen Hospital (Izmir), Başkent University Hospital (Ankara), Koşuyolu State Hospital (Istanbul), and American Hospital (Istanbul) between April 2015 and June 2016. ATCC 17978 standard strain was used as calibrator for qRT PCR. Two colistin susceptible *A.baumannii* clinical isolates (K14, K178) were used for comparing gene sequences in sequence analysis. This study is approved by Koç University Ethical committee and the protocol number is 2015.048.IRB1.008.

2.2. Antibiotic Susceptibility Testing

The minimum inhibitory concentration (MICs) of colistin was determined by E-test (BioMérieux, France) method. Isolates were grown on Tryptic Soy Agar (TSA) (Becton, Dickinson and Company, U.S.) overnight. 10 mL of Mueller Hinton broth (MH) (Becton, Dickinson and Company, U.S.) with a McFarland 0,5 was prepared from each isolate. After bacteria were transferred on MH agar by a sterile swap, E-tests were placed on the agar plates and overnight incubation at 37°C was done. The resistance breakpoint was set as >2 mg/L according to the Clinical and Laboratory Standards Institute (CLSI) guideline[95] .

2.3. Molecular Analysis

Total DNA and RNA isolation from clinical isolates was done in order to use for further molecular analysis. After overnight growth on at 37°C, DNA was extracted with the commercial DNA extraction kit according to the manufacturer's instructions (MoBio UltraCleanMicrobial DNA Isolation 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 8-12 hours to obtain bacteria on logarithmic growth phase. After incubation, density was adjusted to McFarland 1 (~$300x10^6$) 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 genotyping of *A.baumannii* clinical isolates by rep-PCR based diversilab system, PCR and sequencing of *lpxA, lpxC, lpxD* genes and *pmrCAB* operon, carbapenamase typing PCR, and detection of *mcr-1* by PCR. Extracted RNA were used in quantitative real time 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

In order 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 17978 was for calibration.

2.4 Statistical Analysis

Statistical analysis was performed using the R (R Core Team, 2016). In the heat map normalized -ΔΔ signal values of probes and hierarchical clustering with complete linkage to obtain dendrograms of rows and column were used. Wilcoxon rank-sum test (also known as the Wilcoxon-Mann-Whitney test or the Mann-Whitney U test) was used to make statistical comparisons between two samples. All the results of statistical analysis are available at: https://midaslab.shinyapps.io/colistin_resistant_acinetobacter_analysis/

CHAPTER 3

RESULTS

3.1 Demographic Characteristics of Study Population

A total of 29 patients infected with colistin resistant *A.baumannii* were included in the study. All of the patients were hospitalized in the ICU. Fifty eight percent of the patients were female and mean age was 67.1 year (32-88 years). *A.baumannii* isolates were obtained from different sources: 8 (27.5%) respiratory tract, 6 (20.6%) deep tracheal aspiration, 3 (10.3%) bronchial lavage, 3 (10.3%) rectal swap, 3 (10.3%) blood, 2 (6.8%) urine, 1 (3.4%) drain fluid, 1 (3.4%) abdominal, and 1 (3.4%) wound. The mean duration of the colistin therapy was calculated as 8.03 days (0-25 days).

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 colistin resistant clinical isolates carry $bla_{\text{OXA-23.}}$ Figure 3.1 shows the PCR result of samples between Case 25 and Case 32 and as the rest of the isolates, all of them are OXA-23 positive. For positive control, an OXA-23 positive carbapenem resistant *A.baumannii* clinical isolate from a previous study was selected.

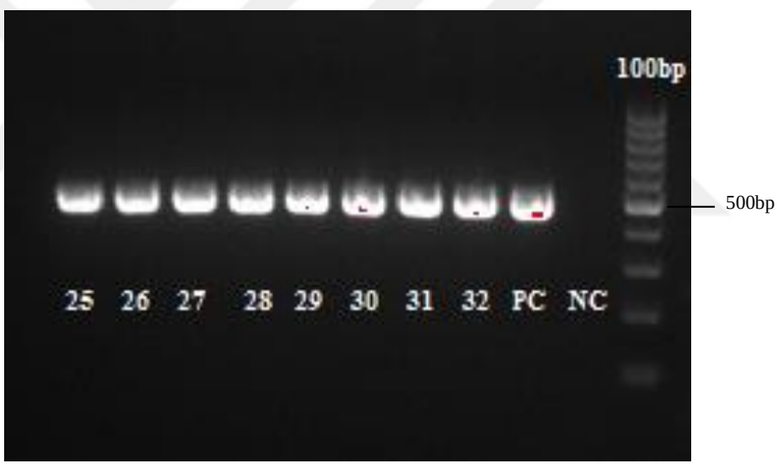

Figure 3.1 Agarose gel image of carbapanamase typing multiplex PCR which detects $bla_{\text{OXA-23,}}$ $bla_{\text{OXA-24}}$, and $bla_{\text{OXA-58}}$ of the samples between Case 25 and Case 32

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 MIC Values

The median colistin MIC50 value of the 29 colistin resistant isolates was 12 μg/ml (range: 2-256 μg/ml).

3.4 Clonal Relatedness of Isolates

The dendogram of the strains isolated from patients who admitted to the different healthcare centers was shown in the Figure 3.2. Regarding to the similarity index, isolates which have more than 95% similarity are accepted as the member of the same clone. Accordingly, Cases 28, 29, 31, and 32 isolated from hospital A form a small clone while isolates Case 22, 15, 13, 11, 10, 8, 17, 9, 23, 21 obtained from hospital B and Case 33 is isolated from hospital A were clustered in another clone.

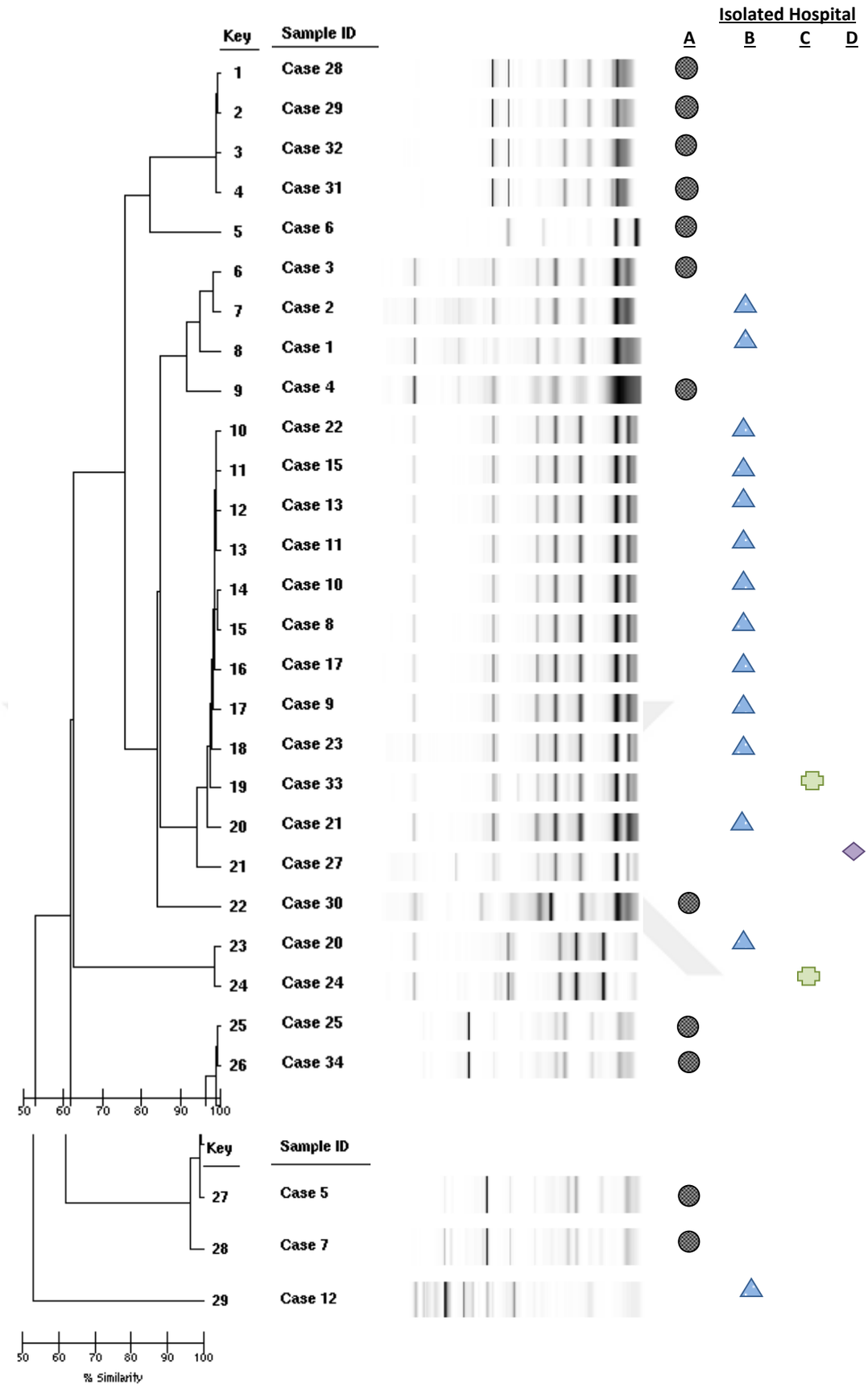

Figure 3.2 Dendogram of 29 colistin resistant clinical isolates. Similarity index scale is on the bottom of the dendogram. Name of the samples illustrated as Case and rep-PCR gel images of these samples were shown next to the dendogram. ●, ▲, ✚, ◆ represent hospitals designated as A, B, C, and D respectively.

3.5 PmrCAB and LpxA, LpxC, LpxD Sequence Analysis

In order to sequence *lpxA, lpxC, lpxD* genes and complete *pmrCAB* operon, regions were sequenced after the amplification by sequence PCR which uses dideoxy-chain termination method. In order to detect mutations in PmrA, PmrB, PmrC, LpxA, LpxC, and LpxD sequence reads were both aligned to ATCC 19606, ATCC 17978 and colistin susceptible clinical isolates; K14 and K178.

No insertions or deletion were detected however, various amino acid variations were found in PmrA, PmrB, PmrC, LpxA, LpxC, and LpxD. In Table 3.2 amino acid changes are presented. The most common amino acid changes were detected T138A in PmrB, V42I& F150L in PmrC, and E117K in LpxD regions.

Table 3.2 Amino acid variations and their positions in the PmrC, PmrA, PmrB, LpxA, LpxC, and LpxD. In the third and last row, mutation after aligning sequences by taking K14&K178 or ATCC 19606 and ATCC 17978 as consensus were presented.

Protein	Mutation	Consensus K14&K178 (%)	Consensus ATCC 19606/17978 (%)
PmrC	V42I	19(65.5)	19(65.5)
	R109H	9(31)	9(31)
	I115N	5(17.2)	5(17.2)
	F150L	19(65.5)	19(65.5)
	N284D	0	29(100)
PmrA	M12I	2(6.8)	2(6.8)
PmrB	H89L	5(17)	5(17)
	T138A	17(58.6)	17(58.6)
	I163F	1(3.4)	1(3.4)
	I164L	1(3.4)	1(3.4)
	A224V	1(3.4)	1(3.4)
	E229D	1(3.4)	1(3.4)
	P233S	1(3.4)	1(3.4)
	R263H	8(27.6)	8(27.6)
	G390H	0	29(100)
	A408E	1(3.4)	1(3.4)
LpxA	Y131H	0	29(100)
LpxC	C120R	0	29(100)
	N287D	0	29(100)
LpxD	Q4K	5(17.2)	5(17.2)
	V63I	5(17.2)	5(17.2)
	E117K	19 (65.5)	19 (65.5)

3.6 *pmrC, pmrA, pmrB Expression* Analyses

Relative expression levels of *pmrC*, *pmrA* and *pmrB* were indicated in Figure 3.3, 3.4, and 3.4 respectively. As it is indicated in the figures, expression levels of the targeted genes were increased in most of the isolates. The mean relative fold increase is calculated as 35.3-fold in *pmrC*, 12.3-fold in *pmrA*, and 8.2-fold in *pmrB*.

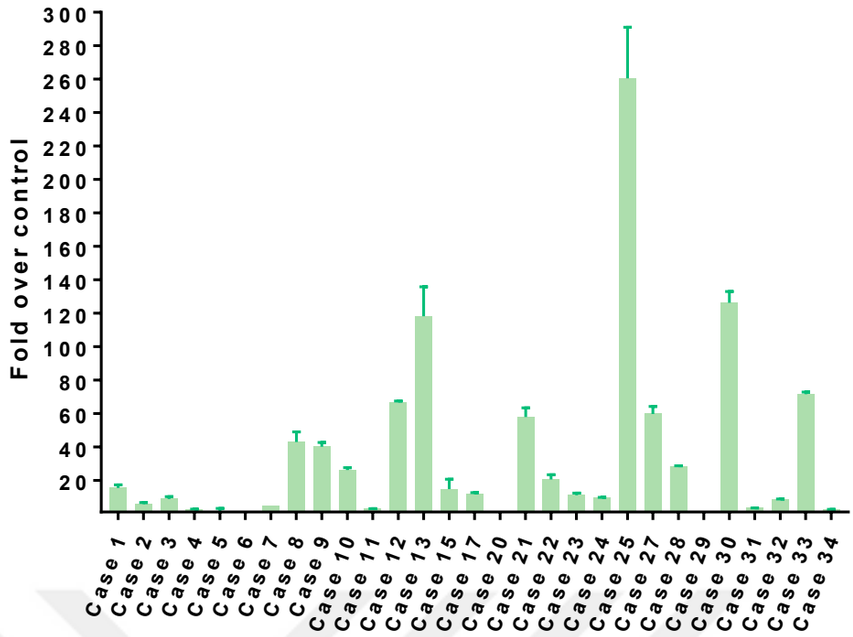

Figure 3.3 Relative expression levels of *pmrC* gene in 29 different clinical isolates. ATCC 17978 was used as calibrator

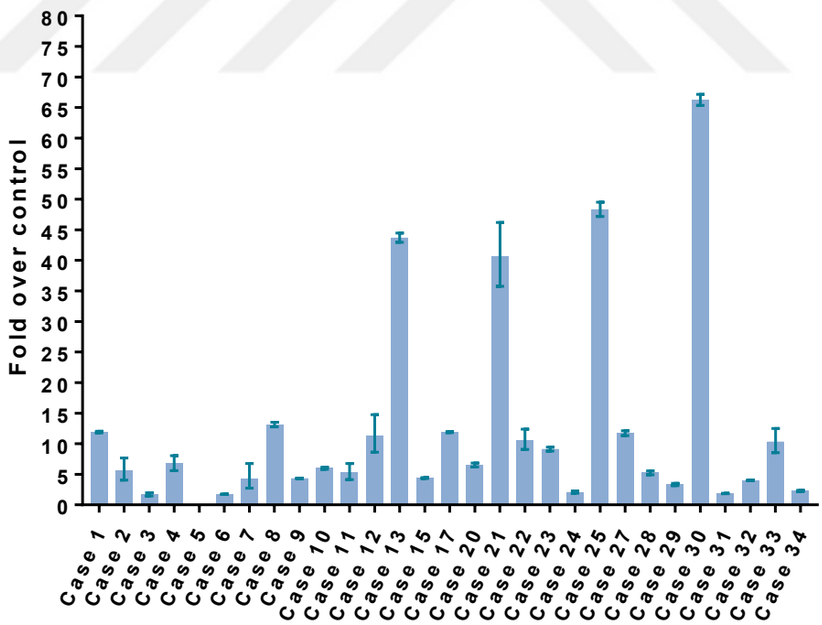

Figure 3.4 Relative expression levels of *pmrA* gene in 29 different clinical isolates. ATCC 17978 was used as calibrator

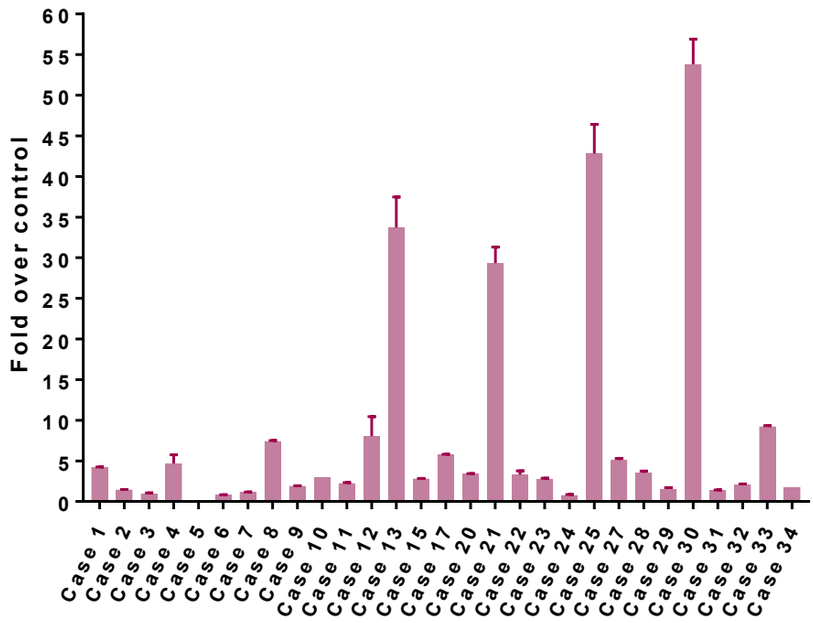

Figure 3.5 Relative expression levels of *pmrB* gene in 29 different clinical isolates. ATCC 17978 was used as calibrator

3.7 The Expression Levels of *pmrCAB* Operon in Correlation with Host Factors, Point Mutations and MIC Values

The association between molecular alterations of *A.baumannii* isolates and demographic data of the patients were demonstrated in the Figure 3.6. A heatmap that combines the expression levels of *pmrC*, *pmrA*, and *pmrB* genes with mutations in PmrA, PmrB, PmrC, LpxA, LpxC, and LpxD proteins and changes in, colistin MIC values, fatality, age, duration of colistin use and having operation in last month data was generated.

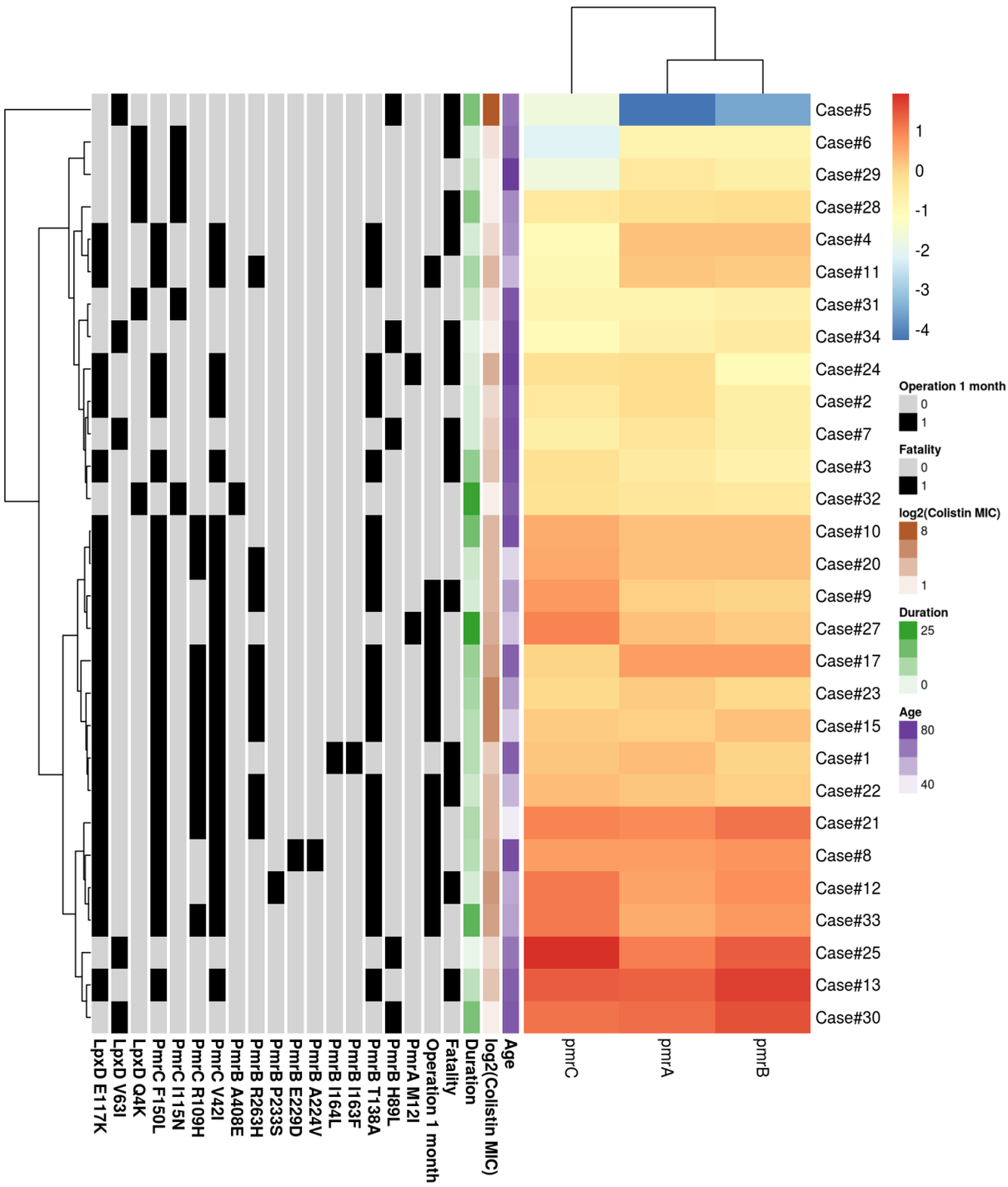

Figure 3.6 Heat map combined with patient's clinical data and biological data of isolates. Amino acid changes in Pmr and Lpx proteins, fatality status, having operation within last 1 month, duration of colistin usage, patient's age, colistin MIC values, and expression levels of *pmrA, pmrB, pmrC,* of the isolates are presented. Cases that have mutations and patients who had operation in last month are represented as black. Colistin administration is in green, log2 colistin MIC values are in brown, and age distribution is designated in purple scale as shown in the right of the heat map

According to the dendogram in the Figure 3.6, gene expression patterns of *pmrA* and *pmrB* were in correlation with each other while *pmrC* expression was more independent.

The duration of colistin therapy was found to be associated with *pmrB* overexpression (p=0.046).(Supplement:https://midaslab.shinyapps.io/colistin_resistant_acinetobacter_analysis/)

As indicated in the Figure 3.7, *A.baumannii* strains isolated from the patients operated within 1 month before colistin resistant *A.baumannii* isolation were significantly associated with expression levels of *pmrB* and *pmrC* (p: 0.039 and 0.044 respectively) The *pmrA* expression was found to be high but non-significant in operated patients (p: 0.055).

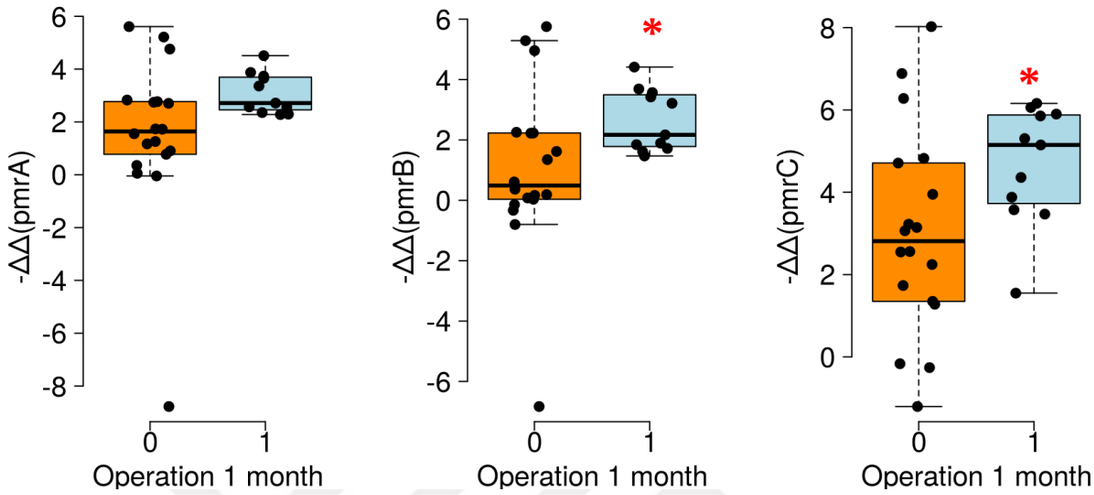

Figure 3.7 The – ΔΔ Ct values of *pmrA*, *pmrB*, and *pmrC* genes correlated with the patient's operation status. The blue bar shows the operated patients, orange bar shows the patients with no operation *, statistically significant

As presented in the Figure 3.8, the MIC values of *A.baumannii* strains isolated from the patients operated within 1 month before colistin resistant *A.baumannii* isolation were significantly higher than the isolates from not operated patient with median values 16 μg/ml vs 4 μg/ml, respectively (p:0.0003).

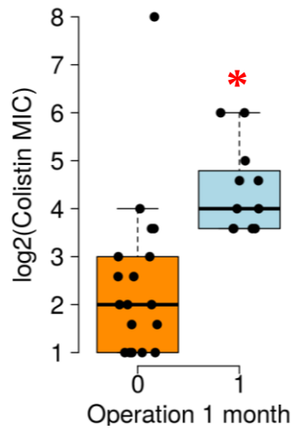

Figure 3.8 The MIC values of isolates compared the with the patient's operation status. The blue bar shows the operated patients, orange bar shows the patients with no operation. *, statistically significant

Figure 3.9 shows the dendogram of expression patterns of the *pmrC*, *pmrA*, and *pmrB* genes combined with the clonal relatedness of the isolates examined by rep-PCR. The isolates which were found to be similar by rep-PCR were distributed along the expression dendogram.

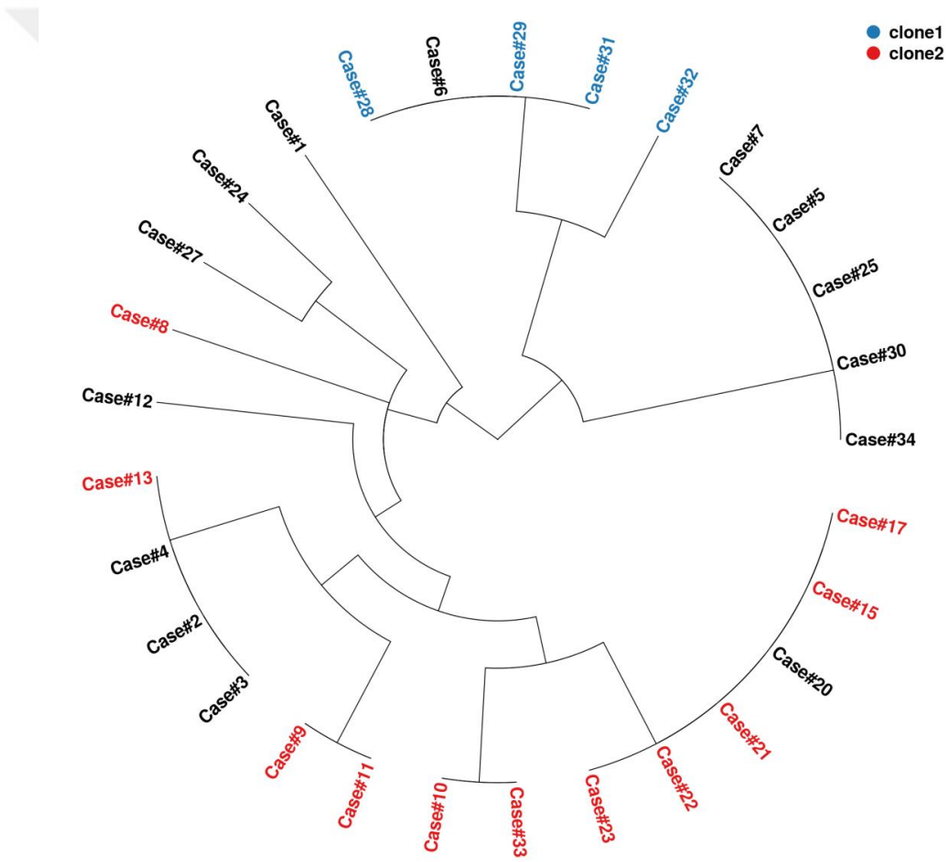

Figure 3.9 Similarity dendogram of *pmrC*, *pmrA*, and *pmrB* expression level patterns. Clone 1 (blue) and clone 2 (red) were major clones identified in the rep-PCR dendogram designated in Figure 3.2

3.8 Correlation between Amino acid Changes and Colistin MIC Values

In the Figure 3.10, amino acid variations with the selection of colistin susceptible clinical isolates K14 and K178 as consensus sequences were demonstrated. According to the Figure 3.9 all isolates have at least 2 amino acid changes in either one of the Lpx or Pmr proteins.

The combination of PmrC V42I, PmrC F150L, and LpxD E117K mutations was detected in the Cases 1, 2, 3, 4, 8, 9, 10, 11, 12, 13, 15, 17, 20, 21, 22, 23, 24, 27, and 33. Among these isolates, 16 of them were accompanied with PmrB T138A mutation, and 8 of them were accompanied with PmrB R263H mutation. As shown in the Fgireu 3.10, all the cases operated within last month were cumulated in these mutation clone. Cases 5, 7, 25, 30, and 34 maintain amino acid changes of PmrB H89L and LpxD V63I together. Also PmrC I115N and LpxD Q4K mutations were combined in Cases 6, 28, 29, 31 and 32 in which only Case 32 possesses additional PmrB A408E variation.

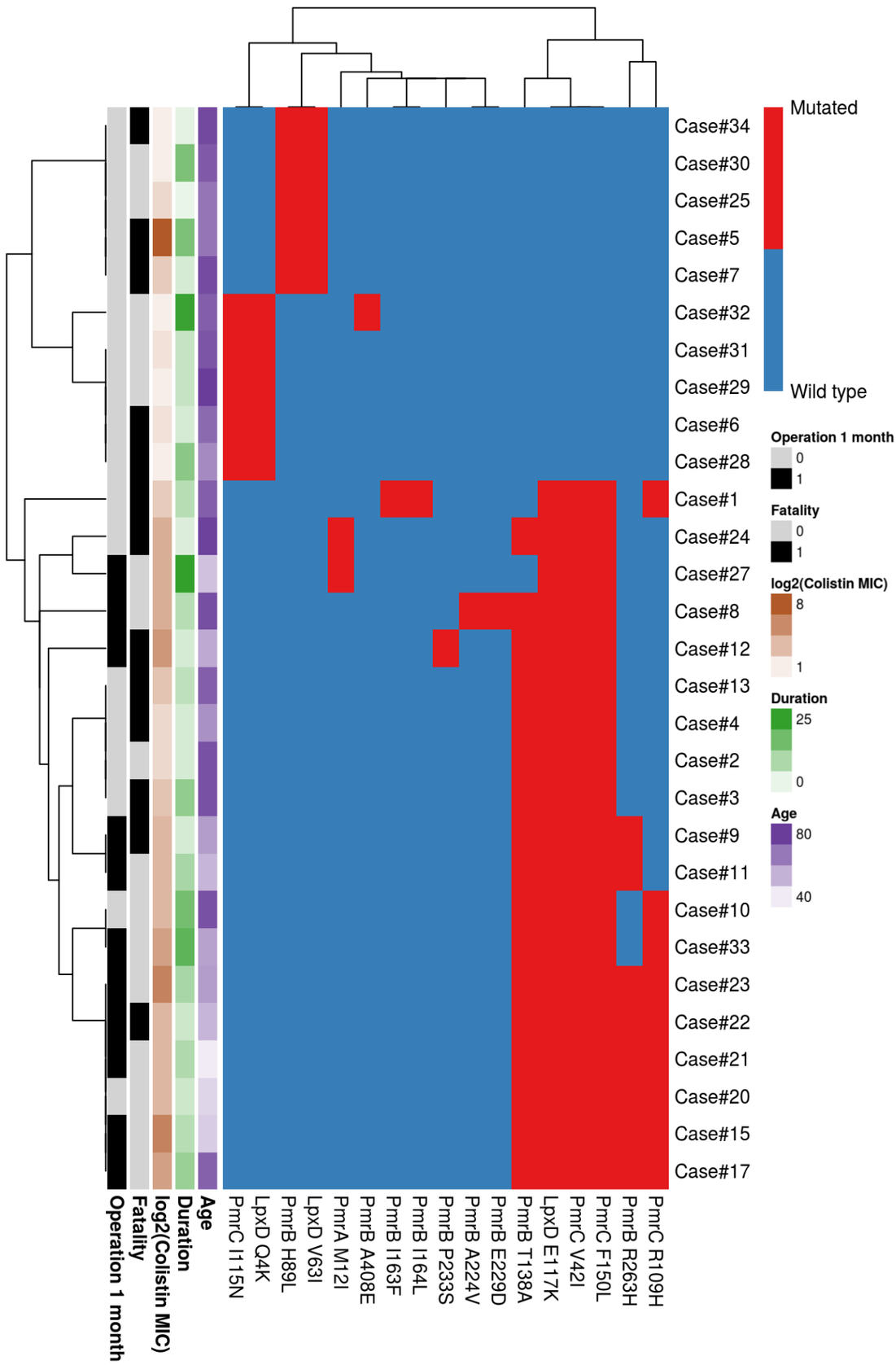

Figure 3.10 Amino acid variations along with the demographic data. Amino acid changes in PmrA, PmrB, PmrC, LpxA, LpxC and LpxD proteins in each Case are presented with red color. On the left, operation in last 1 month before the isolation of colistin resistant *A.baumannii*, and fatality are shown as black. Log2 colistin MIC values are illustrated in brown, duration of colistin administration is in green, and age distribution is in purple scale.

In Figure 3.11, MIC values according to the amino acid variations in PmrC, PmrB, and LpxD are presented.

As shown in Figure 3.11-A PmrC V42I and R109H mutations were found to be significantly associated with the colistin MIC values (p= 0.00086 and 0.017, respectively). The median MICs of isolates with and without mutation in PmrC V42I were 12 μg/ml and 3 μg/ml, and for PmrC R109H median MICs were 12 μg/ml and 6 μg/ml, respectively. Also PmrC F150L mutation was found to be associated with higher colistin MIC levels (median MICs are 12 μg/ml vs 3 μg/ml) (p=0.00086).

As illustrated in Figure 3.11-B, the MIC values of PmrB T138A mutation positive isolates were significantly higher than the isolates without mutation with median values of 12 μg/ml vs 3 μg/ml, respectively (p=0.0022). Similarly, PmrB R263H mutation was found to be effective on high colistin MIC's with a median of 12 μg/ml in positives, and 6 μg/ml in negative isolates (p=0.0022). Also it was found that LpxD E117K mutation is significantly associated with the increase in colistin MIC values (median colistin MIC is 12 μg/ml in positive and 3 μg/ml in negative isolates) (p:0.00086).

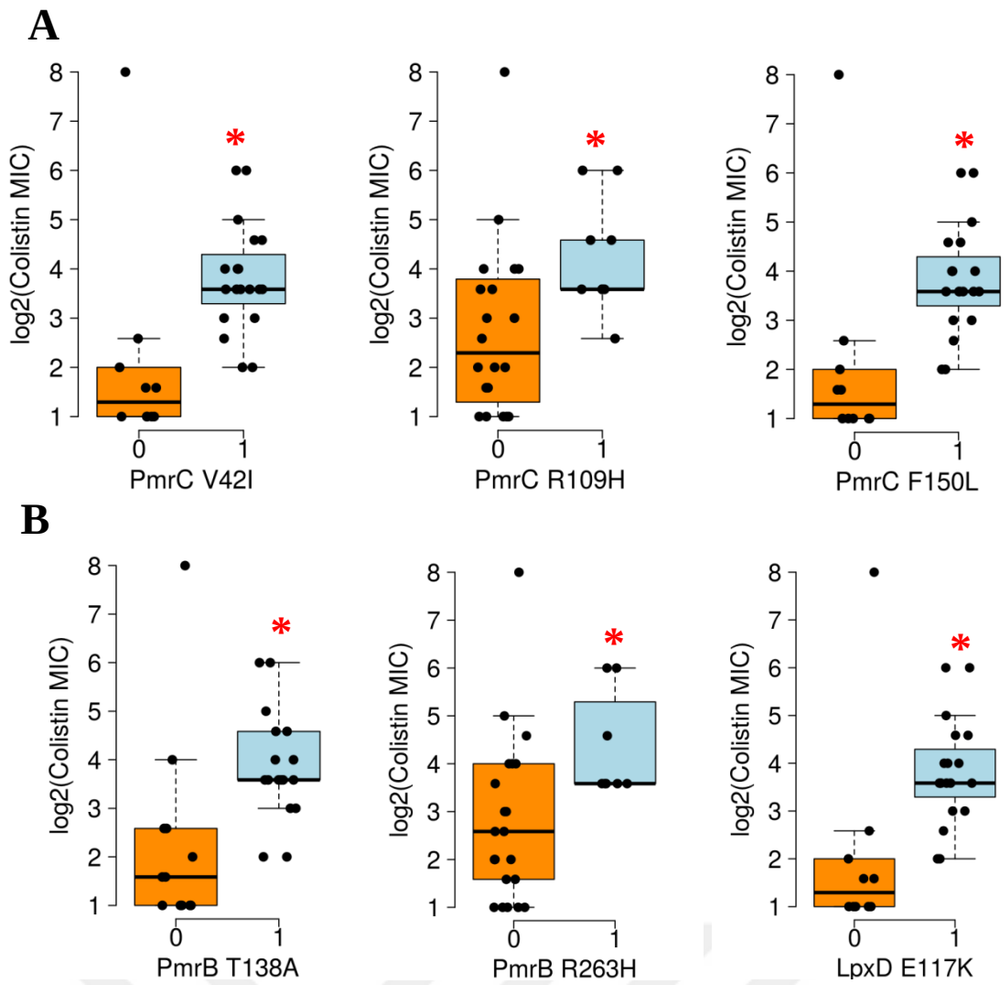

Figure 3.11 The MIC changes with mutation in PmrC,PmrB and LpxD proteins. **A.** PmrC V42I mutations are shown on the left, and PmrC R109H mutations are in the middle. On the left graph PmrC F150L mutations are illustrated. **B.** The MIC changes with mutation in PmrB and LpxD proteins. PmrB T138A mutations are shown on the left graph, and PmrB R263H mutations are in the middle. LpxD E117K mutations are on the right. The orange bars show the isolates with no mutations, blue bars are isolates with mutations.*, statistically significant.

CHAPTER 4

DISCUSSION

Antibiotic resistance is one of the urgent world-wide problem which makes multidrug resistant bacterial infections highly challenging to overcome. Colistin is one of the few options which have been used to treat carbapenem-resistant gram negative bacteria[96]. Unfortunately, reports began to increase describing colistin resistant bacteria and global concern increased due to the recently identified plasmid mediated colistin resistance genes; *mcr-1* and *mcr-2* [91-88]. After the first isolation of colistin resistant *A.baumannii* from Czech Republic in 1999, several surveillance studies reported the colistin resistance rates between 0.9%–3.3%. Later on the finding of high heteroresistance rates in *A.baumannii* revealed that the actual resistance rates are more than anticipated[55].

Up to today several studies were conducted to reveal the genetic mechanisms and relation between mutations and gene expressions. However these studies were performed on laboratory derived colistin resistant mutants or on limited number of clinical isolates without sufficient clinical data[75-80-87-97]. The strongest part of this study is that it includes the largest colistin resistant *A.baumannii* collection accompanied with the clinical data.

The dendogram designated in Figure 3.9 shows that although certain clinical isolates are in same clonal groups, the expression patterns of *pmrC*, *pmrA*, and *pmrB* genes in these isolates can be different. Along the same line, isolates which are not similar to each other may have very similar gene expression patterns. These findings verify the considerable importance of difference in environmental and host factors on gene expression patterns which is the main distinction between laboratory derived colistin resistant isolates and clinical isolates.

qRT-PCR experiments revealed 35.3-fold mean increase in *pmrC* gene. Several studies reported also increased expression levels of *pmrC*; Beceiro et. al, 2011 reported 2.6, 2.1 and 2.8 fold increase and Arroyo et. al., 2011 reported increase between 26 and 292 fold. This wide range of overexpression can be explained by the presence of homologous genes resembles *pmrC* in *A.baumannii*. Lesho et. al., in 2013 identified two homologous genes named *eptA-1* and *eptA-2* in an *A.baumannii* outbreak strain which resembles *pmrC* with >95% homology. Along with the *pmrC*, in this study we also observed 12.3-fold increase in *pmrA*, and 8.2-fold increase in *pmrB* genes as several studies observe the same findings [74-81].

In *S.enterica*, *Yersinia pestis* and *K.pneumoniae* PmrAB two component system is shown to be regulated by PhoPQ system[97]. However in *A.baumannii* regulation of PmrAB two component system is still unclear. Therefore, in this study the dissimilar gene expression patterns of *A.baumannii* isolates in the same clone might be the consequence of different

regulation mechanisms on PmrAB. In order to understand the variations in gene expressions we examined the impact of clinical factors of the patients. Studies so far demonstrated that the colistin usage is the major factor for emergence of chromosomal colistin resistance [98]. In our group except one, all the patients were treated with colistin so we could not examine the effect of colistin exposure on resistance, however we found that *pmrB* expression is significantly associated with the duration of colistin therapy (p:0.046). This situation can explain the accumulation of numerous mutations in PmrB after long term exposure to colistin and these various activating mutations on PmrB might be the reason of increased expression level of *pmrB.*

We also found that operation within last month before the isolation of colistin resistant *A.baumannii* is significantly associated with the overexpression of *pmrC, pmrB* and increased colistin MICs. These results suggest that previously colonized isolates may become colistin resistant and cause invasive infections after surgery.

In this study we detected various mutations in PmrC, PmrA, PmrB and LpxD proteins in colistin resistant *A.baumannii* isolates from the patients in different hospitals. Sequence reads were aligned and compared with both sequenced colistin susceptible clinical isolates K14 and K178, standard calibrator strain ATCC 17978 and sequence obtained from NCBI of ATCC 19606. We identified PmrB G390H, PmrC N284D, LpxA Y131H, LpxC C120R and LpxC N287D amino acid variations when ATCC 17978 and ATCC 19606 strains were used as consensus sequence. These findings reveal that standard calibrator strains and clinical isolates differ in sequences which may be explained by the effect of several environmental factors on clinical isolates. Therefore in the mutation analysis colistin susceptible clinical isolates should be preferred as consensus.

As consistent with the literature the majority of the amino acid changes were detected in PmrB protein. In PmrB, amino acid changes in 9 different locations were observed: H89L, T138A, I163F, I164L, A224V, E229D, P233S, R263H, and A480E. In the PmrB, the active regions of the protein were described between 216-276 HisK and 331-419 HATPaseC[81]. According to this information, the mutations we identified in A224V, E229D, P233S, R263H and A408E are located in the active region of the PmrB. In our study we detected P233S mutation in 1 isolate and R263H mutation in 8 isolates. P233S mutation was described in several studies; Beceiro et. al., in 2011 in 1 of 6 clinical isolates, Arrayo et. al., 2011 in 1 of 10 clinical isolates, Pournaras et. al, 2013 in 1 of 2 clinical isolates, and Kim et. al., 2014 in 10 of clinical isolates [80-81-99-100]. P233S is located in where the dimer formation takes place which may have effect on phosphatase activity of PmrB[80]. The amino acid change in 263. position of PmrB was also described by Arrayo et. al., 2011 in 1 laboratory derived (R263P) and 1 of 6 clinical isolates (R263C), and Lesho et. al., 2013 in 1 of 14 clinical isolates (R263L). Since the changed amino acids in this position varies, this position in PmrB is most likely prone to harbor mutations.

In PmrC, we detected amino acid changes of V42I, R109H, I115N, and N284D in most of our isolates. However there is only one report describing amino acid change in PmrC [81]. Among all the PmrC amino acid variations in our group only N284D was found to be in the sulfatase region which is between aa 237 and 532, other mutations were located in the region that the function is not known [81].

We only observed M12I amino acid change of PmrA in 2 of the isolates which were obtained from 2 different hospitals located in different cities of Turkey (Izmir and Istanbul). This mutation was also described by Arrayo et. al., 2011 in one of the laboratory derived colistin resistant *A.baumannii,* but it was described that the position of this mutation is not within the receiver domain of the PmrA protein[81]. However, detection of this amino acid change in two non-related clinical isolates as seen in the dendogram shown in Figure 3.2, raise the possibility of the active functioning of M12I mutation on colistin resistance in *A.baumannii*.

In LpxA, LpxD and LpxC proteins we determined several amino acid changes only in LpxD: Q4K, V63I, and E117K[84-87]. Since *lpx* genes are involved in the Lipid A biosynthesis pathway, mutations and/or insertions in these genes are expected to create loss of function in protein so that Lipid A synthesis is going to be disrupted. Up to now, different research groups identified several insertions, deletions and mutations caused the function loss of *lpxa*, *lpxc*, and *lpxd* genes but none of the amino acid variations we observed in LpxD were identified before[75-84-87]. However the earlier reports were performed on laboratory derived mutants or low number of clinical isolates. Therefore in our large study group we could detect these mutations. Nevertheless in order to understand the effect of these mutations on LpxD, further experiments revealing the Lipid A structure should be done.

When we compare the mutations and fold changes in expressions of *pmrC*, *pmrB* and *pmrC* genes we found out that the amino acid changes V42I in PmrC, I115N in PmrC, F150L in PmrC is significantly associated with the increased PmrC expression (p:0.031, 0.0085, and 0.031 respectively). Even the most common mutations were detected in *pmrB* region we could not determine an association between these mutations and overexpression in *pmrCAB* operon. However significant association between MIC values and some point mutations in *pmrB,* and *lpxD* regions in our study indicates that the development of colistin resistance is a complex event and probably there are still unidentified factors contribute resistance development.

The carbapanamase typing PCR revealed that all 29 isolates were carrying $bla_{\text{OXA-23.}}$ In Turkey the prevalent type of carbapanamase in *A.baumannii* is OXA-23, and our findings are in parallel with other studies done in our region[101]. We also searched for the presence of plasmid mediated colistin resistance. We could not detect *mcr-1* gene in our isolates as we expected because the lack of *mcr-1* reports in *A.baumannii*. Recently another plasmid encoded colistin resistance gene named *mcr-2* was reported[91]. These rapid findings of new

plasmid supports the possibility of novel, undiscovered plasmids disseminating in *A.baumannii* isolates.

In conclusion, we found out that the expression increase in *pmrC*, *pmrA*, and *pmrB* was involved in colistin resistance of *A.baumannii* but the presence of isolates without overexpression evidences the presence of additional mechanisms contributing to the colistin resistance. The most common mutations in *pmrCAB* operon and *lpxD* gene were found to be associated with high colistin MIC values.

The duration of colistin therapy was associated with the increased *pmrB* expression in *pmrCAB* operon. This connection confirms that the duration of colistin therapy is a substantial factor on emergence of colistin resistance. Therefore, physicians should consider the therapeutic options during colistin treatment by controlling the duration of usage and combining colistin with other antibiotics.

We also found that operation within last month before the isolation of colistin resistant *A.baumannii* is significantly associated with overexpression of *pmrCAB* operon and colistin MIC increase. This finding suggests that alterations in microbiota or depletion of immune system during the surgical applications might trigger colistin resistance and invasiveness of colonized *A.baumannii* isolates. Therefore surveillance cultures, and protective precautions should be taken to prevent the dissemination of resistance.

BIBLIOGRAPHY

1. Qureshi, Z. A. *et al.* Colistin-resistant Acinetobacter baumannii: beyond carbapenem resistance. *Clin. Infect. Dis.* **60,** 1295–303 (2015).

2. HAI Data and Statistics | HAI | CDC. at <http://www.cdc.gov/hai/surveillance/>

3. Revelas, A. Healthcare - associated infections: A public health problem. *Niger. Med. J.* **53,** 59–64 (2012).

4. Sydnor, E. R. M. & Perl, T. M. Hospital epidemiology and infection control in acute-care settings. *Clin. Microbiol. Rev.* **24,** 141–73 (2011).

5. Sydnor, E. R. M. & Perl, T. M. Hospital epidemiology and infection control in acute-care settings. *Clin. Microbiol. Rev.* **24,** 141–73 (2011).

6. WHO | 10 facts on patient safety. *WHO* (2015).

7. Hughes, J. M. Study on the efficacy of nosocomial infection control (SENIC Project): results and implications for the future. *Chemotherapy* **34,** 553–61 (1988).

8. Khan, H. A., Ahmad, A. & Mehboob, R. Nosocomial infections and their control strategies. *Asian Pac. J. Trop. Biomed.* **5,** 509–514 (2015).

9. Horan, T. C., Andrus, M. & Dudeck, M. A. CDC/NHSN surveillance definition of health care–associated infection and criteria for specific types of infections in the acute care setting. *Am. J. Infect. Control* **36,** 309–332 (2008).

10. Mehta, Y. *et al.* Guidelines for prevention of hospital acquired infections. *Indian J. Crit. Care Med.* **18,** 149–63 (2014).

11. WHO | Prevention of hospital-acquired infections: A practical guide. 2nd edition. at <http://www.who.int/csr/resources/publications/drugresist/WHO_CDS_CSR_EPH_2002_12/en/>

12. Joseph, A. Impact of the Environment on Infections in Healthcare Facilities. *Cent. Heal. Des.*

13. Vincent, J.-L. *et al.* The Prevalence of Nosocomial Infection in Intensive Care Units in Europe. *JAMA* **274,** 639 (1995).

14. Vincent, J.-L. *et al.* International Study of the Prevalence and Outcomes of Infection in Intensive Care Units. *JAMA* **302,** 2323 (2009).

15. McKibben, L. *et al.* Guidance on Public Reporting of Healthcare- Associated Infections: Recommendations of the Healthcare Infection Control Practices Advisory Committee •. *Infect. Control Hosp. Epidemiol.* **26,** 580–587 (2005).

16. R.D., S. The direct medical costs of healthcare-associated infections in US hospitals and the benefits of prevention. at <http://www.cdc.gov/hai/pdfs/hai/scott_costpaper.pdf>

17. http://www.who.int/gpsc/country_work/gpsc_ccisc_fact_sheet_en.pdf. at <http://www.who.int/gpsc/country_work/gpsc_ccisc_fact_sheet_en.pdf>

18. Rosenthal, V. D. *et al.* Device-associated nosocomial infections in 55 intensive care units of 8 developing countries. *Ann. Intern. Med.* **145,** 582–91 (2006).

19. Erdem, H. & Akova, M. Leading infectious diseases problems in Turkey. *Clin. Microbiol. Infect.* **18,** 1056–67 (2012).

20. Turkish Society of Clinical Microbiology and Infectious Diseases, H. care related infections study group. Health-care associated Gram Negative Bloodstream Infections: Emergence of Resistance and Predictors of Fatality.

21. Inan, D. *et al.* Daily antibiotic cost of nosocomial infections in a Turkish university hospital. *BMC Infect. Dis.* **5,** 5 (2005).

22. Cisneros, J. M. & Rodríguez-Baño, J. Nosocomial bacteremia due to Acinetobacter baumannii: epidemiology, clinical features and treatment. *Clin. Microbiol. Infect.* **8,** 687–693 (2002).

23. Perez, F. *et al.* Global challenge of multidrug-resistant Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **51,** 3471–84 (2007).

24. Fournier, P.-E. *et al.* Comparative Genomics of Multidrug Resistance in Acinetobacter baumannii. *PLoS Genet.* **2,** e7 (2006).

25. Peleg, A. Y., Seifert, H. & Paterson, D. L. Acinetobacter baumannii: emergence of a successful pathogen. *Clin. Microbiol. Rev.* **21,** 538–82 (2008).

26. Dijkshoorn, L., Nemec, A. & Seifert, H. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. *Nat. Rev. Microbiol.* **5,** 939–951 (2007).

27. Bergogne-Bérézin, E. & Towner, K. J. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin. Microbiol. Rev.* **9,** 148–65 (1996).

28. Neely, A. N. A survey of gram-negative bacteria survival on hospital fabrics and plastics. *J. Burn Care Rehabil.* **21,** 523–7

29. Tomaras, A. P., Dorsey, C. W., Edelmann, R. E. & Actis, L. A. Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system. *Microbiology* **149,** 3473–84 (2003).

30. Rodríguez-Baño, J. *et al.* Biofilm formation in Acinetobacter baumannii: associated features and clinical implications. *Clin. Microbiol. Infect.* **14,** 276–8 (2008).

31. Maragakis, L. L., Perl, T. M. & Perl, T. M. Antimicrobial Resistance: *Acinetobacter baumannii:* Epidemiology, Antimicrobial Resistance, and Treatment Options. *Clin. Infect. Dis.* **46,** 1254–1263 (2008).

32. Pigmenten als oxydatieproducten gevormd door bacterien. at <https://www.google.com.tr/_/chrome/newtab?espv=2&ie=UTF-8>

33. Bouvet, P. J. M. & Grimont, P. A. D. Taxonomy of the Genus Acinetobacter with the Recognition of Acinetobacter baumannii sp. nov., Acinetobacter haemolyticus sp. nov., Acinetobacter johnsonii sp. nov., and Acinetobacter junii sp. nov. and Emended Descriptions of Acinetobacter calcoaceticus a. *Int. J. Syst. Bacteriol.* **36,** 228–240 (1986).

34. Giammanco, A., Vieu, J. F., Bouvet, P. J., Sarzana, A. & Sinatra, A. A comparative assay of epidemiological markers for Acinetobacter strains isolated in a hospital. *Zentralblatt für Bakteriol. Int. J. Med. Microbiol.* **272,** 231–41 (1989).

35. Ruzin, A., Keeney, D. & Bradford, P. A. AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in Acinetobacter calcoaceticus-Acinetobacter baumannii complex. *J. Antimicrob. Chemother.* **59,** 1001–4 (2007).

36. Turton, J. F. *et al.* Identification of Acinetobacter baumannii by detection of the blaOXA-51-like carbapenemase gene intrinsic to this species. *J. Clin. Microbiol.* **44,** 2974–6 (2006).

37. Higgins, P. G., Wisplinghoff, H., Krut, O. & Seifert, H. A PCR-based method to differentiate between Acinetobacter baumannii and Acinetobacter genomic species 13TU. *Clin. Microbiol. Infect.* **13,** 1199–201 (2007).

38. Gonzalez-Villoria, A. M. & Valverde-Garduno, V. Antibiotic-Resistant Acinetobacter baumannii Increasing Success Remains a Challenge as a Nosocomial Pathogen. *J. Pathog.* **2016,** (2016).

39. Goldstein, F. W. *et al.* Transferable plasmid-mediated antibiotic resistance in Acinetobacter. *Plasmid* **10,** 138–147 (1983).

40. Perez, F. *et al.* Global challenge of multidrug-resistant Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **51,** 3471–84 (2007).

41. Manchanda, V., Sanchaita, S. & Singh, N. Multidrug resistant acinetobacter. *J. Glob. Infect. Dis.* **2,** 291–304 (2010).

42. Blechschmidt, B., Borneleit ', P. & Kleber, H.-P. Purification and characterization of an extracellular p-lactamase produced by Acinetobacter calcoaceticus. *J. Gen. Microbiol.* **138,** 197–1202 (1992).

43. Obana, Y., Nishino, T. & Tanino, T. *In-vitro* and *in-vivo* activities of antimicrobial agents against *Acinetobacter calcoaceticus*. *J. Antimicrob. Chemother.* **15,** 441–448 (1985).

44. Naas, Thierry ; Coignard, Bruno ; Carbonne, Anne ; Blanckaert,Karine; Bajolet, Odile ; Bernet, Claude; Verdeil, Xavier; Astagneau, Pascal ; Desenclos, Jean-Claude; Nordmann, P. VEB-1 Extended-Spectrum Beta-Lactamase-Producing Acinetobacter Baumannii, France. *Emerg. Infect. Dis.* 1214–1222 (2006).

45. ABRAHAM, E. P. & CHAIN, E. An Enzyme from Bacteria able to Destroy Penicillin. *Nature* **146,** 837–837 (1940).

46. Fournier, P. E. & Richet, H. The epidemiology and control of Acinetobacter baumannii in health care facilities. *Clin. Infect. Dis.* **42,** 692–9 (2006).

47. Bergogne-Bérézin, E. The increasing significance of outbreaks of Acinetobacter spp.: the need for control and new agents. *J. Hosp. Infect.* **30 Suppl,** 441–52 (1995).

48. Diene, S. M. & Rolain, J.-M. Carbapenemase genes and genetic platforms in Gram-negative bacilli: Enterobacteriaceae, Pseudomonas and Acinetobacter species. *Clin. Microbiol. Infect.* **20,** 831–838 (2014).

49. Corinna Glasner, by *et al.* Carbapenemase-producing bacteria in Europe Interim results from the European survey on carbapenemase-producing Enterobacteriaceae (EuSCAPE) project 2013 Carbapenemase-producing bacteria in Europe.

50. Li, J. *et al.* Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect. Dis.* **6,** 589–601 (2006).

51. Urban, C. *et al.* Polymyxin B-Resistant Acinetobacter baumannii Clinical Isolate Susceptible to Recombinant BPI21 and Cecropin P1. *Antimicrob. Agents Chemother.* **45,** 994–995 (2001).

52. Li, J. *et al.* Heteroresistance to colistin in multidrug-resistant Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **50,** 2946–50 (2006).

53. Reis, A. O., Luz, D. A. M., Tognim, M. C. B., Sader, H. S. & Gales, A. C. Polymyxin-resistant Acinetobacter spp. isolates: what is next? *Emerg. Infect. Dis.* **9,** 1025–7 (2003).

54. Gales, A. C., Reis, A. O. & Jones, R. N. Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J. Clin. Microbiol.* **39,** 183–90 (2001).

55. Cai, Y., Chai, D., Wang, R., Liang, B. & Bai, N. Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. *J. Antimicrob. Chemother.* **67,** 1607–15 (2012).

56. Ergönül Önder. Sağlık Bakımıyla ilişkili Gram Negatif Bakteremilerde Fataliteyi Belirleyen Faktörlerin Araştırılması. in *Ulusal Sağlık Bakımıyla İlişkili İnfeksiyonlar Simpozyumu* (2014).

57. Falagas, M. E., Makris, G. C., Dimopoulos, G. & Matthaiou, D. K. Heteroresistance: a concern of increasing clinical significance? *Clin. Microbiol. Infect.* **14,** 101–4 (2008).

58. Giamarellou, H., Antoniadou, A. & Kanellakopoulou, K. Acinetobacter baumannii: a universal threat to public health? *Int. J. Antimicrob. Agents* **32,** 106–19 (2008).

59. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. *EFSA J.* **13,** 4006 (2015).

60. When the drugs don't work. *Nat. Microbiol.* **1,** 16003 (2016).

61. Falagas, M. E., Kasiakou, S. K. & Saravolatz, L. D. Colistin: The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Clin. Infect. Dis.* **40,** 1333–1341 (2005).

62. Zavascki, A. P., Goldani, L. Z., Li, J. & Nation, R. L. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrob. Chemother.* **60,** 1206–15 (2007).

63. Evans, M. Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant gram-negative bacteria. *Ann. Pharmacother.* **33,** 960–967 (1999).

64. Bergen, P. J. *et al.* Pharmacokinetics and pharmacodynamics of "old" polymyxins: what is new? *Diagn. Microbiol. Infect. Dis.* **74,** 213–23 (2012).

65. Gallardo-Godoy, A. *et al.* Activity and Predicted Nephrotoxicity of Synthetic Antibiotics Based on Polymyxin B. *J. Med. Chem.* **59,** 1068–1077 (2016).

66. Bader, M. W. *et al.* Recognition of Antimicrobial Peptides by a Bacterial Sensor Kinase. *Cell* **122,** 461–472 (2005).

67. Brown, D. A. & Tsang, J. C. Chemical and electrophoretic changes induced by polymyxin B on outer membrane components from Serratia marcescens. *J. Antibiot. (Tokyo).* **31,** 603–9 (1978).

68. Cajal, Y., Rogers, J., Berg, O. G. & Jain, M. K. Intermembrane molecular contacts by polymyxin B mediate exchange of phospholipids. *Biochemistry* **35,** 299–308 (1996).

69. Clausell, A. *et al.* Gram-negative outer and inner membrane models: insertion of cyclic cationic lipopeptides. *J. Phys. Chem. B* **111,** 551–63 (2007).

70. Yu, Z., Qin, W., Lin, J., Fang, S. & Qiu, J. Antibacterial mechanisms of polymyxin and bacterial resistance. *Biomed Res. Int.* **2015,** 679109 (2015).

71. Sampson, T. R. *et al.* Rapid killing of Acinetobacter baumannii by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrob. Agents Chemother.* **56,** 5642–9 (2012).

72. Lopes, J. & Inniss, W. E. Electron microscopy of effect of polymyxin on Escherichia coli lipopolysaccharide. *J. Bacteriol.* **100,** 1128–9 (1969).

73. Falagas, M. E., Rafailidis, P. I. & Matthaiou, D. K. Resistance to polymyxins: Mechanisms, frequency and treatment options. *Drug Resist. Updat.* **13,** 132–8 (2010).

74. Adams, M. D. *et al.* Resistance to Colistin in Acinetobacter baumannii Associated with Mutations in the PmrAB Two-Component System. *Antimicrob. Agents Chemother.* **53,** 3628–3634 (2009).

75. Moffatt, J. H. *et al.* Colistin Resistance in Acinetobacter baumannii Is Mediated by Complete Loss of Lipopolysaccharide Production. *Antimicrob. Agents Chemother.* **54,** 4971–4977 (2010).

76. Mark R. Pelletier, L. G. C. J. W. J. M. D. A. D. V. Z. K. R. O. H. Y. D. R. K. E. Unique Structural Modifications Are Present in the Lipopolysaccharide from Colistin-Resistant Strains of Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **57,** 4831 (2013).

77. Madigan, M. T., Martinko, J. M. & Brock, T. D. *Brock biology of microorganisms*. 34 (Pearson Prentice Hall, 2006).

78. Raetz, C. R. H., Reynolds, C. M., Trent, M. S. & Bishop, R. E. Lipid A modification systems in gram-negative bacteria. *Annu. Rev. Biochem.* **76,** 295–329 (2007).

79. Gunn, J. S. The Salmonella PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol.* **16,** 284–90 (2008).

80. Beceiro, A. *et al.* Phosphoethanolamine modification of lipid A in colistin-resistant variants of Acinetobacter baumannii mediated by the pmrAB two-component regulatory system. *Antimicrob. Agents Chemother.* **55,** 3370–9 (2011).

81. Arroyo, L. A. *et al.* The pmrCAB operon mediates polymyxin resistance in Acinetobacter baumannii ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrob. Agents Chemother.* **55,** 3743–51 (2011).

82. Lesho, E. *et al.* Emergence of colistin-resistance in extremely drug-resistant Acinetobacter baumannii containing a novel pmrCAB operon during colistin therapy of wound infections. *J. Infect. Dis.* **208,** 1142–51 (2013).

83. Pelletier, M. R. *et al.* Unique Structural Modifications Are Present in the Lipopolysaccharide from Colistin-Resistant Strains of Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **57,** 4831–4840 (2013).

84. Moffatt, J. H. *et al.* Insertion sequence ISAba11 is involved in colistin resistance and loss of lipopolysaccharide in Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **55,** 3022–4 (2011).

85. Henry, R. *et al.* Colistin-Resistant, Lipopolysaccharide-Deficient Acinetobacter baumannii Responds to Lipopolysaccharide Loss through Increased Expression of Genes Involved in the Synthesis and Transport of Lipoproteins, Phospholipids, and Poly- -1,6-N-Acetylglucosamine. *Antimicrob. Agents Chemother.* **56,** 59–69 (2012).

86. Chin, C.-Y., Gregg, K. A., Napier, B. A., Ernst, R. K. & Weiss, D. S. A PmrB-Regulated Deacetylase Required for Lipid A Modification and Polymyxin Resistance in Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **59,** 7911–4 (2015).

87. Lim, T. P. *et al.* Multiple Genetic Mutations Associated with Polymyxin Resistance in Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **59,** 7899–902 (2015).

88. Liu, Y.-Y. *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* **16,** 161–168 (2016).

89. McGann, P. *et al.* Escherichia coli Harboring mcr-1 and blaCTX-M on a Novel IncF Plasmid: First Report of mcr-1 in the United States. *Antimicrob. Agents Chemother.* **60,** 4420–1 (2016).

90. Anjum, M. F. *et al.* Colistin resistance in Salmonella and Escherichia coli isolates from a pig farm in Great Britain. *J. Antimicrob. Chemother.* (2016). doi:10.1093/jac/dkw149

91. Xavier, B. *et al.* Identification of a novel plasmid-mediated colistin- resistance gene. *Euro Surveill* **21,** (2016).

92. Beceiro, A. *et al.* Biological Cost of Different Mechanisms of Colistin Resistance and Their Impact on Virulence in Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **58,** 518–526 (2014).

93. López-Rojas, R. *et al.* Colistin resistance in a clinical Acinetobacter baumannii strain appearing after colistin treatment: effect on virulence and bacterial fitness. *Antimicrob. Agents Chemother.* **57,** 4587–9 (2013).

94. Durante-Mangoni, E. *et al.* Emergence of colistin resistance without loss of fitness and virulence after prolonged colistin administration in a patient with extensively drug-resistant Acinetobacter baumannii. *Diagn. Microbiol. Infect. Dis.* **82,** 222–6 (2015).

95. Galani, I. *et al.* Colistin susceptibility testing by Etest and disk diffusion methods. *Int. J. Antimicrob. Agents* **31,** 434–439 (2008).

96. Skov, R. & Monnet, D. Plasmid-mediated colistin resistance (mcr-1 gene): three months later, the story unfolds. *www.eurosurveillance.org* doi:10.2807/1560-7917.ES.2016.21.9.30155

97. Park, Y. K. *et al.* Correlation between overexpression and amino acid substitution of the PmrAB locus and colistin resistance in Acinetobacter baumannii. *Int. J. Antimicrob. Agents* **37,** 525–530 (2011).

98. Wang, Y.-C. *et al.* Risk factors and outcome for colistin-resistant Acinetobacter nosocomialis bacteraemia in patients without previous colistin exposure. *Clin. Microbiol. Infect.* **21,** 758–64 (2015).

99. Pournaras, S. *et al.* Growth Retardation, Reduced Invasiveness and Impaired Colistin-Mediated Cell Death Associated with Colistin Resistance Development in Acinetobacter baumannii 2 3. *Antimicrob. Agents Chemother* (2013). doi:10.1128/AAC.01439-13

100. Kim, Y. *et al.* In vivo emergence of colistin resistance in Acinetobacter baumannii clinical isolates of sequence type 357 during colistin treatment. *Diagn. Microbiol. Infect. Dis.* **79,** 362–366 (2014).

101. Di Popolo, A. *et al.* Molecular epidemiological investigation of multidrug-resistant Acinetobacter baumannii strains in four Mediterranean countries with a multilocus sequence typing scheme. *Clin. Microbiol. Infect.* **17,** 197–201 (2011).