Clinical and Molecular Factors Effecting Colistin Resistance in *K.pneumoniae*

by

Pelin İspir

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

Master of Science
in
Medical Microbiology

Koc University

June 2015
Koc University
Graduate School of Health Sciences

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

Pelin İspir

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:

_____
Füsun Can, M.D. (Advisor)

_____
Önder Ergönül, M.D.

_____
Funda Timurkaynak, M. D.

Date: _____

ABSTRACT

Hospital acquired infections are one of the most important health issue affecting millions of patients and cause significant burden. *Klebsiella pneumoniae (K.pneumoniae)* which is one of the most common agents in worldwide are isolated from nosocomial infections. Colistin has been considered as the last choice of treatment for these infections since carbapenem resistance rate has been increasing in recent years. However, colistin resistance in *K.pneumoniae* began to increase which points out an emerging threat in the treatment of healthcare related infections. *PhoP/PhoQ* and *PmrA/PmrB* are the main systems responsible for modification of LPS layer. Changes in these two component regulatory systems cause colistin resistance. *MgrB* gene, which encodes the key regulatory protein of *PhoP/PhoQ* system, has the major role in the colistin resistance. Insertional inactivation or mutations in *MgrB* gene are highly associated with colistin resistance. In this study; our aim is to understand ongoing emergence of colistin resistance among *K. pneumoniae* strains by analysing the molecular mechanisms of colistin resistance and comparing the demographic risk factors, clinical futures and outcomes of the infections.

A total of 32 patients with a isolation of colistin resistant *K.pneumoniae* from Ankara Başkent University Hospital, İstanbul Başkent University Hospital and Koşuyolu State Hospital were included to the study. Inactivation of *MgrB* gene by transposons and mutations in wild type *MgrB* gene was studied by PCR amplification followed by Sanger sequencing and products were identified by NCBI Blast tool and CLC Genomics Workbench v.4.9 software (CLC Bio, Aarhus, Denmark). Also, the expression levels of *PhoQ* and *PmrK* genes in colistin resistant isolates were analysed by quantitative real time PCR. The clonal analysis of the strains were done by rep-PCR using Diversilab system (bioMérieux, France).The analysis of the clinical and molecular data was done by STATA 11 (Texas, USA).

Insertional inactivation of *MgrB* gene was found in 26 out of 32 isolates. *MgrB* gene was distrupted by different insertion sequences (IS) classified as IS5 like, IS1 like, IS903 like and IS903B. Wild type *MgrB* was seen in 5 isolates. We detected nonsilent point mutations in 4 of the 5 isolates carrying wild type *MgrB*. In one strain we found a point mutation at the position 68, and in one strain we detected a frameshift mutation at the position 9. There was no significant overexpression in *PhoQ* and *PmrK* genes. Our clonal analysis revealed that there were two major clones both containing samples from different centres with >95% similarity. Relatively younger age and staying in intensive care unit were found to be associated with IS related colistin resistance (P<.005). The mean duration of the colistin therapy in IS positive group was longer than the IS negative group (19 versus 7 days, p=.231).

We report the first findings on the molecular and clinical epidemiology of colistin resistance among *K.pneumoniae*. The results of the study show that disruption of *MgrB* gene by transposons is the key mechanism for colistin resistance. Long term stay in intensive care unit, and younger age were major host risk factors that increase the risk of infection by a *K.pneumoniae* strain carrying IS in its *MgrB* gene. Prolonged colistin therapy might have an affect on IS related colistin resistance. Demonstration of the horizontal gene transfer between colistin resistant strains highlights the emergence of colistin resistance as the public health problem of the world.

ÖZET

Sağlık bakımıyla ilişkili enfeksiyonlar her yıl dünyada milyonlarca hastayı etkileyen önemli bir sağlık sorunudur ve ciddi kayıplara yol açar. *K.pneumoniae* dünyada sağlık bakımıyla ilişkili enfeksiyonların en önemli etkenlerinden biridir. Son yıllarda artan karbapenem direnci sebebiyle kolistin en son tedavi seçeneği olarak kullanilmaktadir. Ancak yapılan çalışmalarda kolistin direncinin artmakta olduğu ve önemli bir sağlık tehditi olmaya başladığı bildirilmektedir. *PhoP/PhoQ* ve *PmrA/PmrB* iki komponentli düzenleyici sistemlerdir ve gram negatif bakterilerde kolistin direncine neden olan genlerin ekspresyonlarını kontrol ederler. *MgrB* geni PhoP/PhoQ sisteminin regülatörü olan MgrB proteininini kodlar. Bu gende meydana gelen insersiyonal inaktivasyonlar ve mutasyonlar kolistin direnci ile ilişkilendirilmiştir. Bu çalışmada amacımız hastalardan elde edilen *K.pneumoniae* izolatlarındaki kolistin direncini belirleyen moleküler mekanizmaların klinik verilerle ilişkisini ortaya çıkarmaktır.

Ankara Başkent Üniversitesi Hastanesi, İstanbul Başkent Üniversitesi Hastanesi ve Koşuyolu Devlet Hastanesinde tedavi gören 32 hastadan izole edilen kolistin dirençli K.pneumoniae suşları çalışmaya dahil edilmiştir. *MgrB* geninde transposon vey a mutasyon kaynaklı inaktivasyonun belirlenmesi için PCR amplifikasyonu ve ardından Sanger sekanslama methodu kullanılmıştır. Elde edilen ürünler NCBI'ın Blast aracı ve Genomics Workbench v.4.9 (CLC Bio, Aarhus, Danimarka) programı kullanılarak analiz edilmiştir. Ayrıca kolistin dirençli izolatlarda, *PhoQ* ve *PmrK* gen ifadelerindeki değişimler q-RT PCR metodu ile çalışılmıştır. Suşların klon analizleri rep-PCR yöntemi ile Diversilab Sistemi ile yapılmıştır (BioMérieux, Fransa). İstatistiksel analizler için STATA 11 programı (Teksas, Amerika) kullanılmıştır.

MgrB geninin transposon ile inaktivasyonu 32 izolatın 26'sında gözlenmlenmiştir. Bu transposonlar IS5 benzeri, IS1 benzeri, IS903 benzeri ve IS903B olarak sınıflandırılmıştır. Çalışma populasyonunda, 5 izolatın *MgrB* geninde herhangi bir insersiyon olmayıp , 3'ünde sessiz, 1'inde nokta (T68A), 1'inde frameshift (del9A) mutasyonu bulunmuştur. *PhoQ* ve *PmrK* gen ifadelerinde önemli bir artış gözlemlenmemiştir.

Klon analizi sonucunda üç merkezden toplanan hastaların iki farklı klona ait olduğugörülmüştür (>%95 benzerlik). Relatif olarak genç yaşta olmak, yoğun akım ünitesinde kalmak transposon ilişkili kolistin direncinde rol alan klinik faktörlerdir(P<.005). Ortalama kolistin tedavi süresi transposonla inaktive olmuş *MgrB* pozitif *K.pneumoniae* ile infekte grupta 19 gün iken; transposonsuz *MgrB* grubunda infekte olan grupta 7 gündür (P=.231).

Bu çalışma *MgrB* geninin inaktivasyonuna bağlı kolistin direncinin gelişmesinde rol alan klinik ve moleküler faktörleri gösteren ilk çalışmadır. *MgrB* geninin transposonlar ile inaktivasyonun kolistin direnci kazanılmasında en önemli moleküler mekanizma olduğu gösterilmiştir. Uzun süre yoğun bakım ünitesinde kalma ve daha genç yaşta olma, transposonla inaktive *MgrB* geni bulunan suş ile enfekte olma riskini artırır. Uzun süreli kolistin tedavisinin de bu dirençte etkili olduğu düşünülmektedir. Ayrıca, kolistin dirençli *K.pneumoniae* izolatlarının arasında yatay gen geçişinın gösterilmesi, kolistin direncininin halk sağlığını tehdit eden önemli bir problem olduğunu vurgulamaktadır.

ACKNOWLEDGEMENTS

First of all, I would like to thank to my advisor, Assoc. Prof. Dr. Füsun Can, for giving me the opportunity to undertake my Masters studies in her laboratory, for her guidance, encouragement, patience and sharing her great knowledge throughout this period of time.

Secondly, very special thanks to Prof. Dr. Önder Ergönül, for his constant support, precious advice and excellent supervision. He highlighted my research findings, helped me to put them into a greater context and sharpened the clinical perspective of my research.

I would like to thank to Dr. Funda Timurkaynak, Dr. Şirin Menekşe and Dr. Özlem Azap for their great collaboration in collecting samples from hospitals. Also, thanks to Nathan Lack, for his generosity for helping in molecular analysis part of my thesis.

I acknowledge Koç University for the financial supports during my studies. I am grateful for the scientific and personal experiences I had in Koç University.

I would like to thank to all my laboratory members for their precious helps and advices which has made things easier for me.Special thanks and gratitude to all our Infection Control Nurse Team at Amerikan Hospital and Koç University Hospital. Thank you for great support and a good working climate.

Thanks to my dear friends Ezgi Kaya, Zeynep Kaya, Fatma Özgün, Uğur Gaftar, Çağrı Kurt, Filiz Şenbaboğlu, Hilal Saraç, Müge Atış, Cansu Üretmen, Ahmet Cingöz, Kerem Fidan, Fidan Şeker, Ezgi Özyerli, Eda Süer, Can Aztekin, Görkem Özyurt, Gizem Şahin and all the rest whom I could not mention here for supporting me in any aspect during the completion of this thesis and making my life colorful and enjoyable. Also, special thanks to Hüseyin Aktaş for his unvaluable patience and sincerity.

Last but not least, thank you my parents Elif and Galip İspir for all your love, care, understanding and unfailing support. You always believed in me and allowed me to follow my ambitions. Tülin and Zeynep, my dear sisters, you were in here always with your positive energy. Your love always be secret of my success.

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AST	Antibiotic Susceptibility Testing
AUC	Area Under The Curve
CLSI	Clinical and Laboratory Standards
CHF	Chronic Heart Failure
CRKP	Colistin Resistant K.pneumoniae
DM	Diabetes Mellitus
EDTA	Ethylenediaminetetraacetic Acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HAI	Hospital Acquired Infections
ICU	Intensive Care Unit
IS	Insertional Sequences
KPC	Klebsiella pneumonia Karbapenamase
LB agar	Luria-Bertani agar
MIC	Minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration required to inhibit the
MIC_{90}	growth of 50% of organisms
NCBI	Minimum inhibitory concentration required to inhibit the
NDM	growth of 90% of organisms
OXA	National Center for Biotechnology Information
PBS	New Delhi Metallo-β-lactamase
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid

q-RT PCR	Quantitative Reverse Transcriptase PCR
SPS	Serum Physiologic Solution
TAE BUFFER	Tris Acetic Acid EDTA Buffer
TE BUFFER	Tris Ethylenediaminetetraacetic Acid Buffer
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1 HOSPITAL ACQUIRED INFECTIONS

A hospital acquired infection (HAI)- also called nosocomial infection, can be defined as an infection acquired in hospital or other health care facility which does not present in patient at the time of admission accoding to World Health Organization (WHO)[1].They occur within 48 hours of hospital admission, 3 days of discharge or 30 days of an operation [2]. Although the technological progress in clinical services is continuing, these infections are still one of the major cause of high mortality and morbidity among patients and staff [3,4].

HAIs do not only affect the life quality of patients. Increased use of drugs, needs of isolation, additional laboratory and diagnostic studies are the financial burden of them. On average 37000 individual die every year in Europe because of HAI. This infections result in additional cost of 5.5 million € [5].

HAIs infections occur worldwide with a range between low-income countries and high-income countries. According to WHO, 30 % patients from intensive care units (ICU) in high-income countries have at least one nosocomial infection[1]. Whereas this frequency is at least 2─3 fold higher in low- and middle-income countries. Many studies show that incidence of HAIs are high in developing countries [6].

Inadequate environmental hygienic conditions and waste disposal, poor infrastructure, insufficient equipment, overcrowding, poor knowledge and poor application of basic infection control measures, lack of procedure, absence of local and national guidelines and policies are the major factors causing nosocomial infections[1]. Simple, low cost and

effective methods can reduce the incidence number of these infections [7]. Otherwise nosocomial infections could be epidemic and affect the community as well.

The most common nosocomial infections are infections of surgical wounds, urinary tract infections, lower respiratory tract infections and bacteraemia [8]. Infection rates are higher among patients with old age, underlying disease, or chemotherapy treatment. New-borns are the highest risk groups with 3-20 times more infection rates[1].

Some procedures, such as biopsies, endoscopic examinations, catheterization, intubation/ventilation increase the risk of infection[9]. Because of high usage of antimicrobial agents, resistance emerges to these drugs and spreads in the health care setting[10]. This is an important problem in developing countries where more expensive second-line antibiotics may not be available or affordable [11].

Turkey is a developing country located on the way between Europe, Asia, and Africa. This geographical placement has been responsible for the spreading of many infectious diseases occurring throughout the country. Nosocomial infections comprise an important health-care problem in Turkey, mainly in university hospitals. The hospital prevalence of healthcare-associated infections was reported to be 13.4%, reaching 48.7% in intensive care units [12]. In world, the most common agents are methicillin resistant *S.aureus* (MRSA), vancomycin resistant Enterecoccus (VRE) and gram negatives. However in Turkey, *Acinetobacter baumannii (A.baumannii), Klebsiella pneumoniae (K.pneumoniae), Escherichia coli (E.coli)* and *Pseudomonas aeruginosa (P.aeruginosa)* are mostly predominant [13]. A study reveals that mortality ratio of 594 patients with bacteraemia is 47%. Responsible species are *Acinetobacter baumanniii, Klebsiella pneumonia,* E.*coli*, *Pseudomonas aeruginosa*, with a ratio of 31, 27, 24, 9%, respectively[14]. Apparently outbreak strains have disseminated to other European countries from Turkey [12].

1.2 K.PNEUMONIAE

The genus Klebsiella is a member of Enterobacteriacea family. It takes its name from German Microbiologist Edwin Kleps, includes non-motile, Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharide-based capsule [15]. **Figure 1** shows the cell morphology of *K. pneumonia*. They grow well on standard laboratory medium without specific growth requirements. The optimum temperature of growth is between 35 and 37 °C and at pH 7.2. They are facultative anaerobes, and most strains use citrate and glucose as their sole carbon sources and ammonia as their sole nitrogen source [16].

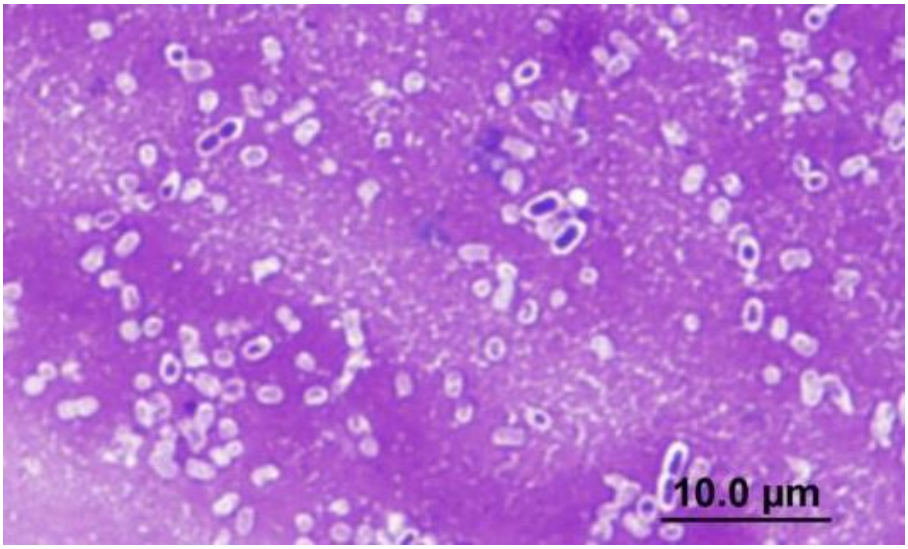


Figure 1. Encapsulated *Klebsiella pneumoniae* stained with Anthony's capsule stain [17]

The most important Klebsiella species in clinic is *K.pneumoniae* [18] As a commensal bacterium, *K.pneumoniae* is found in human gastointestinal tract, eyes, respiratory tract and genitourinary tract[19].

K.pneumoniae infections can range from mild urinary tract infections to severe bacteraemia and pneumonia with a high rate of mortality and morbidity [20]. In some countries *K.pneumoniae* is also one of the major causes of community acquired infection [21].

Since the early 1970s the epidemiology of *K.pneumoniae* has changed dramatically. It is highyl found in colonization stage on patient's nasopharynges, hands and gastrointestinal tracts. This enables it to spread in health care settings easily [22]. An emergence of extended-spectrum beta-lactamase and carbapenemase-producing strains enhances spread and outbreaks of infection.

Many factors affect *K. pneumoniae* pathogenicity. These factors include: 1) the ability to attach or react to the cell through receptors, 2) the ability to protect the bacteria from phagocytosis and to alter the immune response through capsular polysaccharide, and 3) the ability to directly and indirectly modify the cellular immune system to enhance pathogenicity[23].

The outcomes of host-bacteria interactions determine the virulence of *K.pneumoniae*. Mainly two components are important for the virulence of this pathogen, lipopolysaccharide (LPS) and capsular polysaccharide (CPS).LPS consists of lipid A, core, and O-polysaccharide antigen that are critical for the microorganism to escape from complement-mediated killing. **Figure 2** shows the structural representation of LPS layer of *K.pneumoniae* and **Figure 3** shows the molecular parts of it.

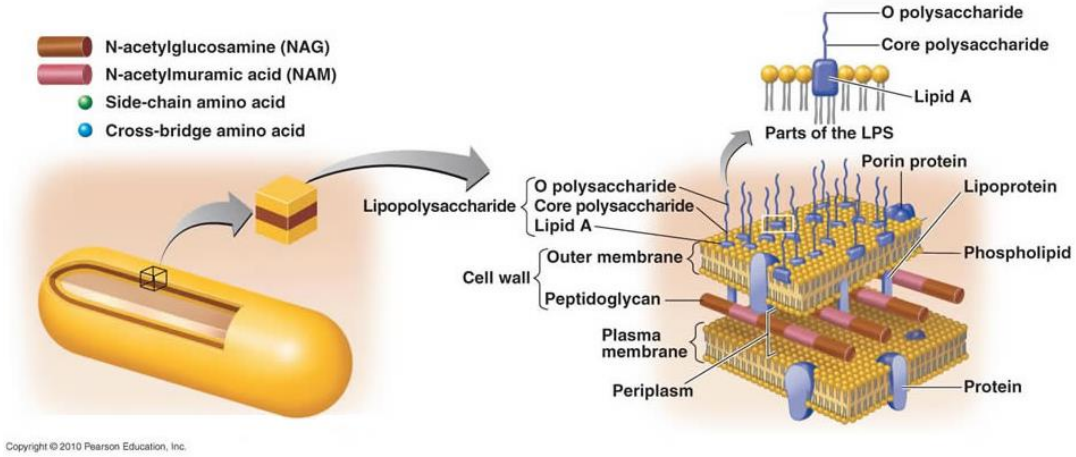


Figure 2. The cell wall structure of *K.pneumoniae* [24]

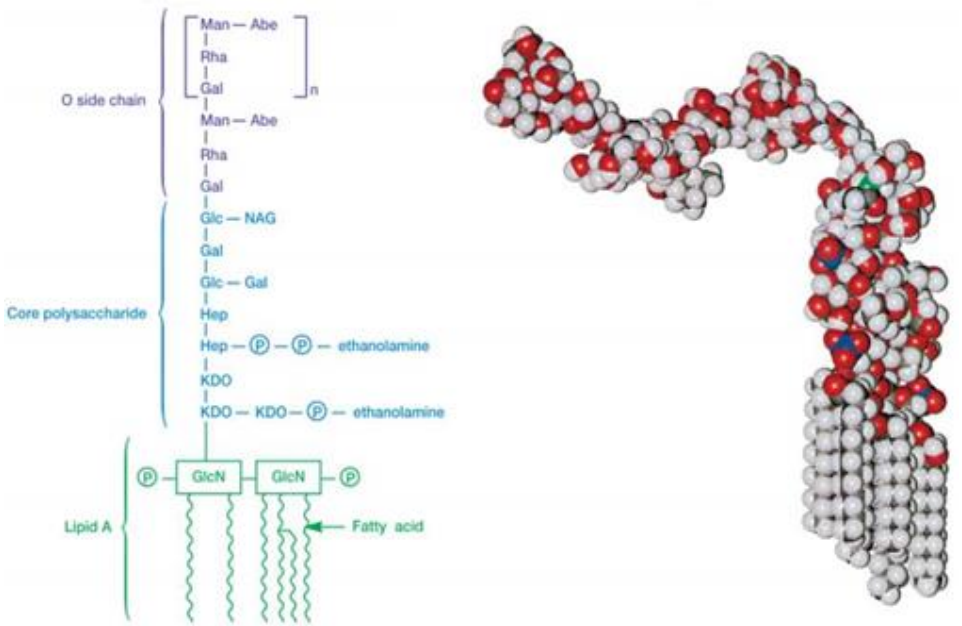


Figure 3. Molecular parts of lipopolysaccharide structure [24]

CPS is the outermost layer of this pathogen. The large polysaccharide capsule and slime that surround the cell envelope protect the cell from phagocytosis by acting as a physical barrier. Therefore, CPS is largely responsible for its survival and capacity to cause disease[20]. In addition to LPS and CPS, the presence of cell wall receptors, endotoxin activities iron-scavenging systems fimbrial and non-fimbrial adhesins are the other key factors in virulence [15,25]. An electron microscopy image of the capsule structure of *K.pneumoniae* is shown in **Figure 4**.

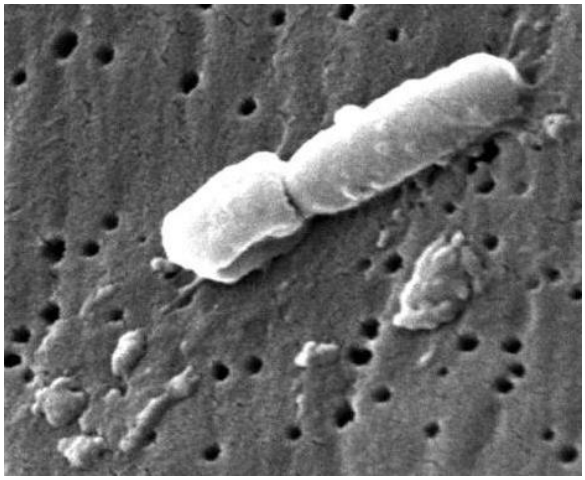


Figure 4. Electron microscopy image of *K.pneumonie* capsule structure [26]

Extended spectrum beta lactamase (ESBL) producing Klebsiella species cause infections with high severity [3]. In the 1980s, carbapenem antibiotics were used as the last line of defense against multidrug-resistant Gram-negative organisms [27]. However, high level carbapenem-resistant KPC-producing bacteria may be selected, during imipenem and meropenem therapy [28]. Moreover, KPC-producing organisms can confer resistance to all available β-lactams, fluoroquinolones, and aminoglycosides [29].

The main carbapenemases found in Enterobacteriaceae are the Ambler class A KPC enzymes, Ambler class B b-lactamases (metallo-b-lactamases) of VIM, IMP and NDM types, and Ambler class D carbapenemases of the OXA-48 type [30]. **Figure 5** shows transposon Tn4401 which carries carbapenemase gene.

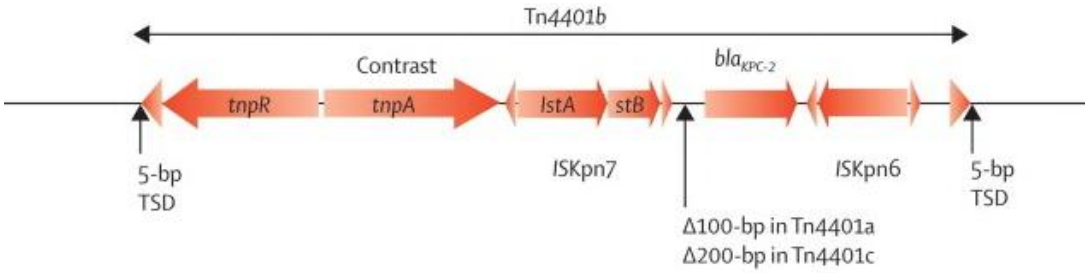


Figure 5. Schematic representation of Tn*4401* structures identified on naturally occurring plasmids [28]

In **Table 1** common β-lactamases found in Klebsiella clinical isolates were shown. In Turkey the most common carbapenamase type is OXA-48 in *K.pneumoniae*[31,32]. NDM-1 type betalactamase was also reported in 2012 in Turkey [33].

After carbapenamases, combination treatment with carbapenems and polymyxins are used for treatment of infections caused by KPC-producing bacteria. Many studies show that monotherapy has higher treatment failure than combination therapy[29]. Unfortunately, in this decade colistin resistance among KPC *K.pneumoniae* has been emerging [34].

Table 1. β-lactamases commonly found in *Klebsiella* clinical isolates [35]

Class	Gene product		Substrate range	Geographic distribution
	Abbreviated name	Expanded name		
Ambler class A (serine-β-lactamase)	TEM-1	Temoneira (patient name) β-lactamase	Primarily penicillins, including ampicillin	Worldwide. Most common plasmid-borne β-lactamase in the *Enterobacteriaceae*
	SHV-18	Sulfhydryl variant ESBL	Penicillins; oxyimino-β-lactams; aztreonam	Worldwide
	CTX-M	Cefotaxime-hydrolyzing ESBL	Broad-spectrum cephalosporins and monobactams; no activity against cephamycins or carbapenems	Worldwide
	KPC	*Klebsiella pneumoniae* Carbapenemase	Penicillins, cephalosporins; lower but clinically relevant activity against carbapenems, aztreonam; low activity against cephamycins and ceftazidime	Worldwide, especially the US, South America, China, Israel, Greece, and Western Europe
Ambler class B (metallo-β-lactamase)	VIM	Verona integron-encoded metallo-β-lactamase	All β-lactamases except monobactams (aztreonam)	Worldwide, especially Asia-Pacific region, including Australia, India, the Philippines, Japan, and China
	IMP	Imipenemase		
	NDM	New Delhi metallo-β-lactamase		Worldwide, especially India, Pakistan, Bangladesh, and the Balkans
Ambler class D (serine-β-lactamase)	OXA-48	Oxacillinase	Penicillins; carbapenems (low but clinically relevant activity); some narrow-spectrum cephalosporins; no activity against oxyimino-cephalosporins	Turkey, Western Europe, North Africa, and the Eastern Mediterranean

1.3 COLISTIN

Polymyxins are derived from *Bacillus polymyxa* subspecies *colistinus* and active only against Gram-negative bacteria. The clinically available forms are polymyxin B and colistin (also known as polymyxin E)[36]. **Figure 6** shows the structure of colistin.

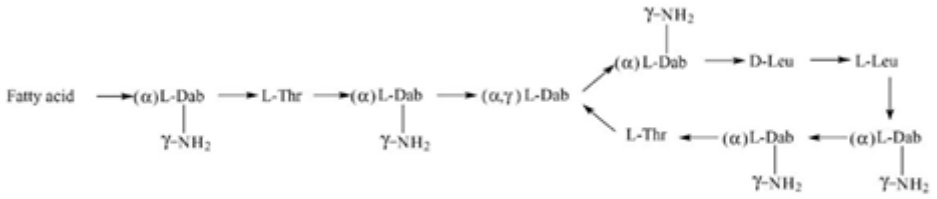


Figure 6. Structure of colistin [37]

Colistin was first introduced in 1952 and was used until the early 1980s to treat gram negative bacilli infections. Because of its nephrotoxicity and availability of second and third generation cephalosporins, colistin was no longer preferred [38].

Colistin is a pentacationic cyclic lipodecapeptide composed mainly of colistin A and B. Like its polymyxin counterpart, colistin has bactericidal action against most gram negative bacteria except bacteria of the genera Proteus, Providencia, Morganella, Serratia, Edwardsiella and Burkholderia, which are known to be intrinsically resistant to colistin.

Colistin exhibits amphipathic properties and able to distribute well in both in water and in prokaryotic and eukaryotic lipid membranes [37]. It interacts with the lipid A moiety of the Gram-negative bacterial lipopolysaccharide (LPS). The polycationic peptide ring competes for and substitutes the calcium and magnesium bridges stabilizing the LPS. This promotes membrane permeability and disrupts the integrity of the outer membrane of Gram-negative bacteria, so leads to bacterial death [39]. A unique anti-endotoxin activity is also a beneficial characteristic of colistin to neutralize bacterial LPS [37]. Colistin exhibits a concentration-dependent bactericidal activity. Schematic representation of colistin acting mechanism , and its therapeutic efficacy strictly depends on the ratio of peak level to minimum inhibitory concentration (MIC) or the ratio of area under the curve (AUC) to MIC [40]. Despite the toxicity of colistin, it is used to treat infections due to carbapenem-resistant *Enterobacteriaceae as* the last resort treatment option [41,42]. Colistin resistance occurs through selection pressure rather than by horizontal transmission. The duration of colistin treatment is an important factor in the emergence of resistance[43]. Although the emergence of colistin resistant *K.pneumoniae* (CRKP) in vivo is likely associated with prior colistin exposure in humans, there are reports of CRKP in patients without any history of colistin therapy [28,44].

There are many reports revealing colistin resistance in Enterobacteriaceae from different parts of world. In Italy colistin incidence ratio is 18.6% in the carbapenem resistant *K.pneumoniae* isolates collected between 2007-2010 [45]. Also, this ratio is 36.1% in intensive care units of Italy [46]. In Turkey colistin resistance ratio of *P.eroginosa* and *K.pneumoniae* are 1.7% and 6% respectively [14]. The colistin resistance ratio of *A.baumannii* is below 7% in the Asia, Europe and United States, however it was reported in the studies from Bulgaria and Spain as 16.7% and 40%, respectively [47]. In Turkey colistin resistance ratio of *A.baumannii* is 5% [14].

Combination therapy with tigecycline and gentamicin revealed less mortality rates than colistin monotherapy [48,49], colistin resistant *K.pneumoniae* outbreaks are emerging and they accounts for high mortality [46,50].

1.4 RESISTANCE MECHANISMS

The key issue in the polymyxin resistance is the alterations of LPSs which are the initial targets of them [47]. These alterations include addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) by covalent modifications of the lipid A moiety of LPS [51]. One of the most frequent LPS modification is the cationic substitution of the phosphate groups by L-Ara4N, which causes a decrease in the net negative charge of lipidA to 0 [52]. The net positive charge of the modified LPS reduces its binding to polymyxins which resuts in resistance.

There have been an extensive search of mechanisms underlying the polymyxin resistance in *K.pneumoniae*. It is observed that in a genetically uncharacterized polymyxin-resistant strain of *K. pneumoniae* the phosphate groups of lipid A contains five times more L-Ara4N than the susceptible strain [44]. These structural modifications of LPS in *K.pneu moniae* occurs via involvement of *PhoP/PhoQ* and *PmrA/PmrB*[53].

Expression of LPS modifying genes depend on the constitutive activation and subsequent overexpression of two-component systems (TCS) involving *PmrA/PmrB* and *PhoP/PhoQ*. These activation and overexpression of TCSs could occur via environmental stimuli or specific mutations of LPS- modifying genes [54].

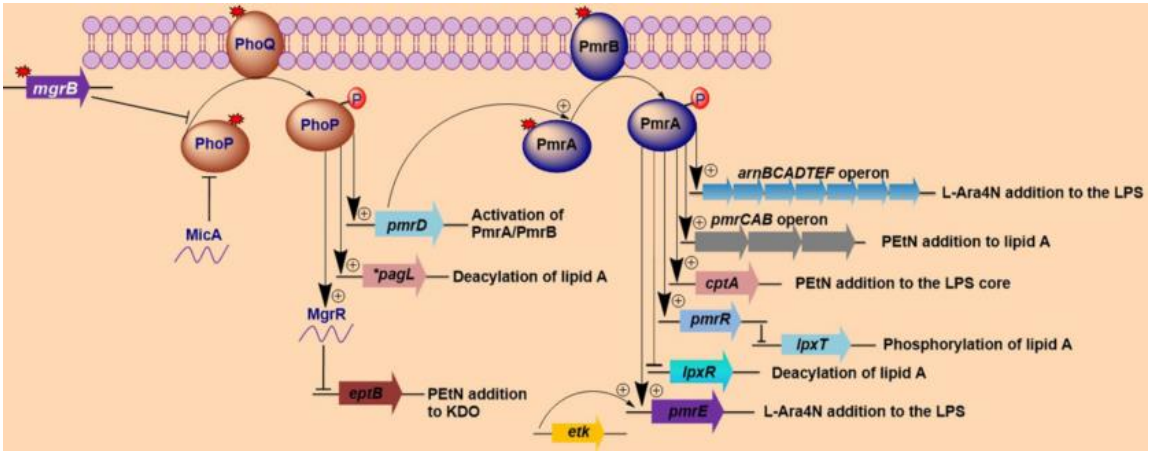


Figure 7. Activation of lipopolysaccharide-modifying genes responsible for polymyxin resistance in *K.pneumoniae*. The mgrB protein provides negative feedback to the *PhoP/PhoQ* regulatory system, while mutations (denoted by red-colored star symbols) in *mgrB* or *PhoP/PhoQ* results in the constitutive induction of the *PhoP/PhoQ* two-component system. The *PmrA/PmrB* two-component system is activated via *pmrD* (which is activated by *PhoP*) or through mutations in the *PmrA/PmrB* genes. After induction, the phosphorylated *PmrA* activates the *arnBCADTEF* and *pmrE* genes, which collectively modify LPSs. Also, the *PhoP/PhoQ* regulatory system can directly activates the *arnBCADTEF* operon[44]

The activation of the *PmrA/PmrB* TCS induces the upregulation of the *pmrCAB* and *arnBCADTEF-pmrE* (also called *pmrHFIJKLM-ugd*) operons that responsible for the synthesis and transfer of PEtN and L-Ara4N to lipid A [51]. There are also missense mutations in *PmrA* or *PmrB* cause upregulation of *pmrC* and the *arnBCADTEF* operon

resulting in the synthesis and addition of PEtN and L-Ara4N, respectively, to lipidA, as shown in **Figure 7**. Both clinical and non-clinical isolates of colisitin-resistant *K.pneumoniae* have different mutations in their *PmrA* and *PmrB* genes [39,44,45]. Some of the mutations that may lead to colistin resistance are shown in **Table 2** [44].

Table 2. Mutations in *PmrA* and *PmrBPmrA* genes of *K.pneumoniae*.

PmrA	***PmrBPmrA***
G53C	L82R
	T157P
	S85R
	T140P
	ΔR14
	ΔY209
	T157P
	S208N

The *PhoQ/PhoP* TCS contributes to polymyxin resistance by indirectly activating the *PmrA/PmrB* TCS via PmrD [55–57]. After activation, PmrA binds to the promoter region of the *arnBCADTEF* operon, increasing the recognition and binding of RNA polymerase and resulting in the upregulation of the operon [58]. Possible mutations in *PhoQ* genes that result in resistance have also been observed in colistin-resistant *K. pneumoniae* [44].

One recent molecular mechanism that leads to the emergence of colistin resistance in *K. pneumoniae* is the mutation/inactivation of the *mgrB* gene [34]. *mgrB* is a conserved gene of 141 nucleotides in length (the length varies in naturally colistin resistant Enterobacteriaceae) encoding a short, 47-aminoacid transmembrane protein. The major role of mgrB protein is to provide negative feedback on the *PhoP/PhoQ* regulatory system.

mgrB carry out this feedback by inhibiting the kinase activity of *PhoQ* and/or stimulating its phosphataseactivity, which later suppresses PhoP phosphorylation, leading to the repression of PhoP-regulated genes (**Figure 7**) [59].

Various disruptions in *mgrB* have recently been described in diverse clinical and non-clinical isolates of colistin- resistant *K. pneumoniae* involving insertional inactivation by an IS5-like element, IS903-like element and other insertion sequences (Figure 8A)[34,39,44,60,61].

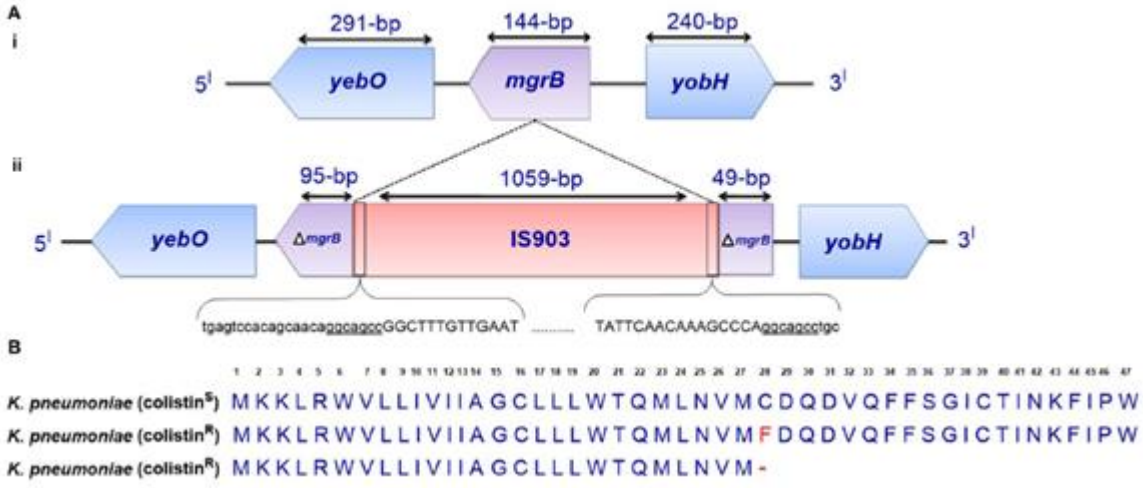


Figure 8. (A) Genetic representation of the *PhoP/PhoQ* negative regulator, *mgrB*. (i) *K. pneumoniae* with intact *mgrB* (colistin-susceptible), and (ii) *K. pneumoniae* with *mgrB* truncated by an insertion sequence (colistin-resistant). **(B)** Alignment of *mgrB* gene of susceptible and resistant *K.pneumoniae* strains. An unmutated *mgrB* from colistin-susceptible *K. pneumoniae* and mutated *mgrB* from a colistin-resistant strain with a missense mutation and premature termination of mgrB. [62].

A non-sense mutation leading to the premature stop codon and therefore malfunctional mgrB transmembrane protein and missense mutations resulting in amino acid substitutions in *mgrB* **(Figure 8B**) [36,45,62]. Several insertion sequences, such as IS5-like, IS903B,IS1F-

like and ISKpn14 elements have been observed to lead to the truncation of *mgrB*. An IS5D-like which is localized on the plasmid initially, has been observed to move chromosome by transposons and inactivates *mgrB* [45,62]. Additionally, various amino acid substitutions, such as C28F which has been reported to affect *PhoQ* activity in *E.coli* [59] have been described in colistin-resistant *K. pneumoniae* [44,45]. Furthermore, small or complete deletions in *mgrB* locus has been reported in various colistin-resistant strains [44,45]. Complementation experiments were done and resulted in the restoration of colistin sensitivity. This disruption of *mgrB* can lead to the upregulation of the *arnBCADTEF* operon, which adds L-Ara4N to lipidA [34]. Compared to TCS mutations (*PmrA*/*PmrB* or *PhoP*/*PhoQ* distruption of *mgrB* responsible for greater colistin resistance among resistant *K. pneumoniae*) [44].

Chapter 2

MATERIALS AND METHODS

2.1 Patient Selection

The study population consisted of 32 adult patients from three different centers in Turkey, Başkent University Hospital, Okmeydanı Hospital and Koşuyolu Hospital with *K.pneumoniae* isolation at any body site during a- 1-year period from June 2014 to May 2015. All the isolates were transported to Koç University School of Medicine Clinical Microbiology Laboratory for molecular identification of colistin resistance. Ethical approval was obtained from Koç University Ethical committee with a protocol number of 2015.048.IRB1.008

2.2 Identification of *K.pnemoniae* Isolates and Antibiotic Susceptibility Testing

The identification of *K.pneumoniea* was performed by automatized systems or conventional techniques. Antibiotic susceptibility testing (AST) was performed using the disc diffusion method on Mueller–Hinton medium (BioMérieux, Marcy l'Etoile, France) and the results were interpreted using Clinical and Laboratory Standards (CLSI) guidelines[63]. Minimum inhibitory concentrations (MICs) for colistin were determined by E-test (BioMérieux, Marcy l'Etoile, France) method. MIC breakpoint for resistance was >2 mg/L[63].

The non-replicate 32 colistin resistant *K.pneumoniae* isolates were stored at -80ºC until the molecular testing for colistin resistance.

2.3 Molecular Analysis of *K.pneumoniae* Isolates

Molecular analysis of the isolates includes amplification of *mgrB* region by polymerase chain reaction (PCR) method, identifying carbapenamase genes (KPC, OXA-48, NDM) by PCR method, sequencing of different sized *mgrB* region with the dideoxy-chain termination method and measuring the expression.levels of *PmrK* and *PhoQ* genes by quantitative real time PCR (qRT-PCR), and determination of the relatedness of the isolates by repPCR.

Chromosomal DNA was extracted using a DNA extraction kit (MoBio UltraClean Microbial DNA Isolation Kit, US). Total RNA was extracted using a RNA extraction kit (Qiagen RNeasy Mini Kit and RNeasy Protect Mini Kit, Germany). Strains subcultered into LB agar and incubated for 12-16 at 37ºC. Ten colonies were picked and inoculated into LB medium, incubated at 37º 8 hours at 37ºC in shaking incubator at 125 rpm. The colonies were homogenized in isotonic saline physiologic solution (SPS) to adjust the turbidity to McFarland 3 (Approximately 10^8 cells). The bacteria were collected by centrifugation 20 minutes at 4500 rcf at 4ºC. Pellets were resuspended and disrupted in TE buffer (pH 8.0) including lysozyme (1 mg/ ml). RNA was stored at -80 ºC until used. Nanodrop measurements were done with Thermo Scientific Nanodrop 2000 Spectrophotometer instrument.

2.3.1 PCR Experiments

All of the genes in this study were amplified using Applied Biosystems Veriti 96 Well Thermal Cycler instrument.

2.3.1.A PCR of Carbapenamase Genes

A multiplex PCR was done to detect each of the three carbapenemase genes (KPC, OXA-48, and NDM) as described by Poirel et al[64]. The carbapenamase genes were amplified with the primers described before. Target genes, primers, product sizes and melting temperature of PCR are given in **Table 3.** Template DNA was subjected to multiplex PCR in a 25-µL reaction mixture. The mix contains 1.5 mmol/L of $MgCl_2$, 2 mmol/L of each deoxynucleotide triphosohate, 25 pmol of each primer and 0.5 U of Phusion DNA polymerase (Biolabs, Germany)

Amplification was carried out with the following thermal cycling conditions: 10 min at 94 C, there were 36 cycles of 94 C, 30 sec.; annealing temperature 60 C, 30 sec.; and 72 C, 50 sec. followed by a final elongation of 5 min at 72C. DNA fragments were analyzed by electrophoresis in a 2 % agarose gel at 100 V for 1h in 1X TAE (40 mmol/L Tris, 20 mM acetic acid,1 mmol/L EDTA).

Table 3. PCR primers and product sizes of carbapenamase genes

Primer	Sequence (5´ to 3´)	Product (bp)
OXA-48-F	GCG TGG TTA AGG ATG AAC AC	
OXA-48-R	CAT CAA GTT CAA CCC AAC CG	438
NDM-F	GGT TTG GCG ATC TGG TTT TC	
NDM-R	CGG AAT GGC TCA TCA CGA TC	621
KPC-F	CGT CTA GTT CTG CTG TCT TG	
KPC-R	CTT GTC ATC CTT GTT AGG CG	798

2.3.1.B PCR of *mgrB* Gene

PCR amplification of the *mgrB* locuses of 32 samples was carried out using primers mgrB-Ext-F and mgrB-Ext-R. These primers target the *mgrB* coding sequence and some

flanking regions. A set of primers covering a larger portion of the *mgrB* locus, and a set of primers targeting amplification of an internal region of the *mgrB* coding sequence were also used. **Figure 9** shows the schematic representation of the gene with different primer sets. A susceptible clinical isolate (crk0) was used as a control strain carrying wild-type *mgrB* in PCR mapping experiments. **Table 4** shows the detailed information of the primer sets used.

Table 4. Primer sets of PCR reactions targeting *mgrB* locus

Primer	Sequence (5´ to 3´)	Product (bp)	Annealing Tm (ºC)
mgrB-EE-F	GGC TAT GGC GAG GAT AAT GAG		
mgrB-EE-R	GCT GTG ATG TAA GCG TCT GGT G	1507	66
mgrB-Ext-F	AAG GCG TTC ATT CTA CCA CC		
mgrB-Ext-R	TTA AGA AGG CCG TGC TAT CC	253	54
mgrB-Int-F	CGG TGG GTT TTA CTG ATA GTC A		
mgrB-Int-R	ATA GTG CAA ATG CCG CTG A	110	54

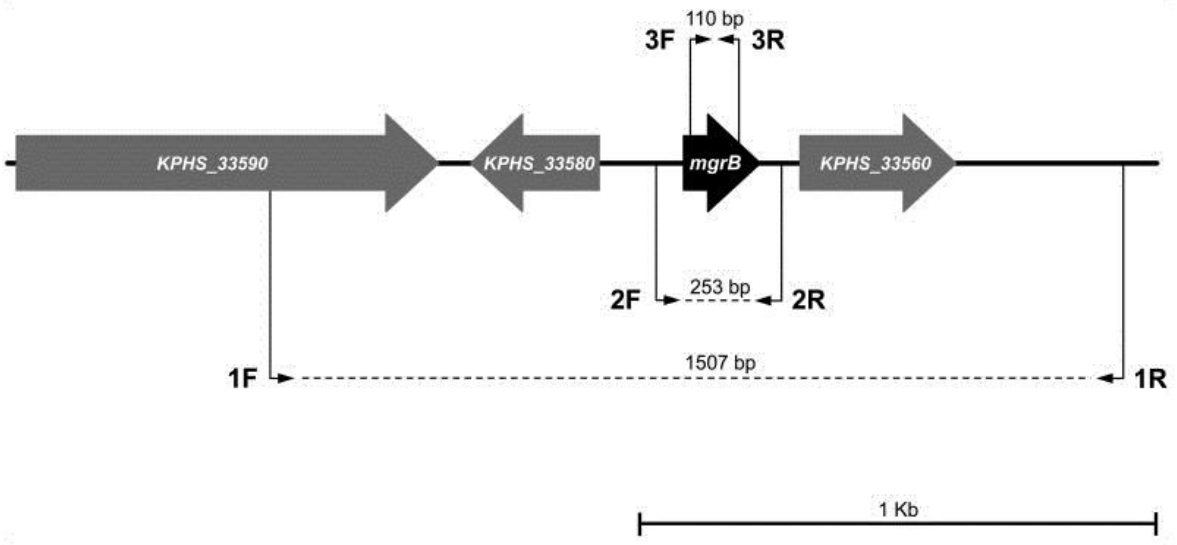


Figure 9. *mgrB* gene environment [34]**.** Three different primer sets used to identify *mgrB* region, namely mgrB-EE (1F and 1R), mgrB-Ext (2F and 2R), mgrB-Int (3F and 3R).

To amplify **253** bp- *mgrB* region, 0.5 microliters of template DNA was subjected to PCR in a 25-µL reaction mixture. The mix contains 6 mmol/L of $MgCl_2$, 2 mmol/L of each deoxynucleotide triphosohate (Thermoscientific, US), 1M betaine, 25 pmol of mgrB-Ext-F and mgrB-Ext-R primers, 0.25 U of Phusion DNA Polymerase (Biolabs, Germany). Amplification was performed under the following conditions: initial denaturation at 95 °C for 3 min, followed by 95 °C for 30 sec, 54 °C for 30 sec and 72 °C for 105 sec (35 cycles), ending with incubation at 72 °C for 5 min. DNA fragments were analyzed by electrophoresis in a 1.5 % agarose gel at 100 V for 1h in 1X TAE Buffer (40 mmol/L Tris, 20 mM acetic acid, 1 mmol/L EDTA).

To amplify **110** bp- *mgrB* region, PCR was performed with 0.5 µl DNA template, 1.5 mM $MgCl_2$, 2 mM of each deoxynucleotide triphosohate (Thermoscientific, US) and 0.25 U Phusion DNA Polymerase (Biolabs, Germany). 25 pmol of mgrB-Int-F and mgrB-Int-R oligonucleotide primer pairs used to amplify the internal region of *mgrB* gene.

Amplification was performed under the following conditions: initial denaturation at 95°C for 3 min, followed by 95 °C for 30 sec, 54 °C for 30 sec and 72 °C for 30 sec (30 cycles), ending with incubation at 72 °C for 5 min. DNA fragments were analysed by electrophoresis in a 1.5 % agarose gel at 100 V for 1h in 1X TAE (40 mmol/L Tris, 20 mM acetic acid, 1 mmol/L EDTA).

To amplify **1507** bp- *mgrB* region, 0.5 microliters of total DNA was subjected to PCR in a 25-µL reaction mixture. The mix contains 10 mM $MgCl_2$, 2 mM of each deoxynucleotide triphosohate (Thermoscientific, US) and 0.25 U Phusion DNA Polymerase (Biolabs, Germany). Amplification was performed under the following conditions: initial denaturation at 95 °C for 180 sec, followed by 95 °C for 30 sec, 66 °C for 30 sec and 72 °C for 120 sec (30 cycles), ending with incubation at 72 °C for 300 sec. DNA fragments were analysed by electrophoresis in a 1.5 % agarose gel at 100 V for 1h in 1X TAE (40 mmol/L Tris-HCl [pH 8.3], 2 mmol/L acetate, 1 mmol /L EDTA).

Samples that had mgrB-Ext band (at 253 bp) in their gel image were purified and \subjected to sequencing experiments, others were amplified by mgrB-EE PCR and then sequenced.

2.3.2 Sequencing Experiments

All of the PCR products were analysed by electrophoresis in 1.5-2.0% agarose gel to detect specific amplified product by comparing with standard molecular weight markers (GeneRuler, Thermoscientific, US). PCR products were purified using a PCR purification kit (Macherey-Nagel NucleoSpin Gel and PCR Clean-up, Germany). For all sequencing experiments, temperature conditions of cycle sequencing PCR was initial heating at 96 ºC 1 minute, 25 cycles of 96ºC for 10 sec, 50ºC for 5 sec and 60ºC for 4 minutes. Sequencing of the PCR products was performed with the dideoxy-chain termination method by Applied

Biosystems ABI 3500 Genetic Analyzer. The extension products were purified by Zymo Research DNA Sequencing Clean-up Kit, USA.

2.3.2.A Carbapenamase gene sequencing

In order to verify multiplex PCR results, one sample (crk17) which is positive for OXA-48 gene were purified and sequenced. In sequencing PCR, Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) were used. Cycle sequencing PCR mix included 2µl of Big Dye, 1µl of Big Dye Terminator 1.1/3.1 Sequencing Buffer, 12.8 µl distilled water, 1 pmol of forward and reverse PCR primers (**Table 2**) and 1 µl of purified PCR product per sample.

2.3.2.B *mgrB* gene sequencing

In sequencing PCR, 1 pmol forward and reverse mgrB- ext primers were used (**Table 2**). The sequence of a 144-bp portion of the *mgrB* gene was established on both strands by using primers mgrB-Ext-F and mgrB-Ext-R, which amplify an internal portion of 123 bp of the gene. For one sample (crk25) mgrB-Ext-F and mgrB-Ext-R primers amplified no gene, therefore with mgrB-Int-F/R and mgrB-EE- F/R primers used for PCR amplification and sequencing of the *mgrB* locus. The nucleotide sequences were analysed at the National Center for Biotechnology Information (NCBI) web site (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool program and Bionumerics Version 7.5. ISs were analysed using the IS finder web site (www-is.biotoul.fr) and CLC Genomics Workbench v.4.9 software (CLC Bio, Aarhus, Denmark). *K.pneumoniae* plasmid 1084 carrying wild type *mgrB* gene (Gene Bank Accession Number: NC_016845) as used as reference sequence.

2.3.3 Quantitative Reverse Transcriptase PCR (qRT-PCR) Experiments

For cDNA synthesis, 500 ng of total RNA in 15.8 µl reaction volume was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) for qRT-PCR. Until used the cDNA was stored at -20ºC.

LightCycler 480 SYBR Green I Master mix was used as a fluorescent dye in expression experiments. A 25 µl reaction mixture included 10 µl of LightCycler 480 SYBR Green I Master mix, 1 µl of 10 pmol forward and reverse primers and 3 µl of distilled water. The protocol of RT-PCR experiments is given at **Table 6**.

Transcription levels of *PmrK* and *PhoQ* were analyzed using Roche LightCycler 480 II instrument. Gene specific primers were used in reaction (**Table 5**). *rpsl* gene was used as housekeeping reference gene. All amplifications were done in duplicate and normalization was done with *K. Pneumoniae* reference strain ATCC 700831.

Table 5. qRT-PCR primer sets

Primer	Sequence (5´ to 3´)
Rpsl-F	CCG TGG CGG TCG TGT TAA AGA
Rpsl-R	GCC GTA CTT GGA GCG AGC CTG
PmrK-F	CGC TGA ATA TGC TCG ACC CAG AAG
PmrK-R	GCT GGC GGT AAT CGT CTG TAC G
PhoQ-F	ATA TGC TGG CGA GAT GGG AAA ACG G
PhoQ-R	CCA GCC AGG GAA CAT CAC GCT

2.3.4 Rep-PCR Experiments

The DiversiLab Microbial Typing System (BioMérieux, Marcy l'Etoile, France) was used to analyse bacterial genomes with repetitive PCR method.. The DNA concentration was set between 25 ng/µl and 30 ng/µl. Later the repetitive sequence elements were amplified using the DiversiLab fingerprinting kit for *Klebsiella* spp. according to the manufacturer's instructions. PCR was performed using the following cycling conditions: initial denaturation (94°C) for 2 min, and then 35 cycles of 30 s of denaturation (94°C), 30 s of annealing (55°C), and 90 s of extension (70°C), followed by 3 min of final extension

(70°C) and ending at 4°C. The amplification products were separated with the Agilent B2100 Bioanalyzer. Five microliters of DNA standard markers (used for normalization of sample runs) and 1 µl of the DNA product were used. All data were entered in the DiversiLab Microbial Typing software system.

Table 6. q-RT PCR protocol for *Rpsl*, *PhoQ* and *PmrK* Genes

Setup				
Detection Format	**Block Type**		**Reaction Volume**	
SYBR Green	96		20 µl	
Programs				
Program Name	Cycles		Analysis Mode	
Pre-incubation	1		None	
Amplification	30		Quantification	
Melting Curve	1		Melting Curves	
Cooling	1		None	
Temperature Targets				
Target (ºC)	**Acqusition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (ºC/s´)**	**Acqusition (per ºC)**
Pre-incubation				
95	None	00:05:00	4.4	-
Amplification				
95	None	00:00:10	4.4	-
65	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	-

2.4 Statistical analysis

Statistical analysis was performed using STATA 11 (Texas, USA). Differences were considered statistically significant at *P*-values of ≤ 0.05. Chi square test were used for comparing the categorical variables, and t-test were used for continuous variables. Univariate and analyses for the prediction of the risk factors of colistin resistance determined by logistic regression. The independent variables were, age, sex, ICU stay, duration of colistin therapy, chronic heart diseases, diabetes mellitus, carbapenamase production, *PmrK* and *PhoQ* expression in the model.

Chapter 3

RESULTS

3.1 Study population

The mean age of the patients included in this study was 58± 21. 1 (15 - 91) and 70% of them was female. Sixty two percent (62%) of the patients were treated in the intensive care unit (ICU). The total fatality rate was 50% whereas the fatality rate in intensive care unit was 70% (P=.003). Among the patients, 15.6% had chronic heart failure (CHF). Colistin was used for the empirical or agent specific treatment of 22 (69%) patients. The duration of colistin therapy was maximum 56 days (mean 14 days). Empirical colistin therapy was used for 6 of the patients (mean 16 days). Agent specific colistin therapy was applied to 16 patients for 15 days in average. The six of the 10 patients who did not receive colistin were in colonization stage, infection did not diagnosed.

3.2 Microbiological Identification and Antibiotic Susceptibilities

The majority of samples were collected from deep tracheal aspirate (50 %). Antibiotic resistance of the strains to most of the antimicrobials tested was over 50%. The resistance ratios were shown in **Table 7.** MIC values of the isolates to colistin were between 3 and 256 μg/ml. MIC_{50} was 16 μg/ml, MIC_{90} was 24 μg/ml. In 12 (38%) of 32 isolate multi drug resistance (MDR) were observed. MDR is defined as being resistant to at least one agent in three or more different classes of antibiotics.

Table 7. Antibiotic resistance (%) of colistin resistant strains by disc diffusion method.

	Resistance	
Antimicrobial Agent	**No(n)**	**%**
meropenem	19	59.3
tigecycline	2	6.3
sulbactam-ampicillin	16	50
ceftriaxone	16	50
ciprofloxacin	16	50
gentamicin	12	37.5
colistin	32	100

3.3 Molecular Detection and identification of Carbapenamase Gene

In total, 22 out of 32 (69%) samples were positive for carbapenemase enzyme. All carbapenemase positive isolates were subjected into multiplex PCR which amplifies KPC, OXA-48, and NDM-1 genes. All of the isolates possessed OXA-48 type carbapenemases. **Figure 10** shows agarose gel electrophoresis image of one experimental panel. Selected one OXA-48 positive isolate was confirmed by sanger sequencing. None of the isolates had KPC and NDM-1 type carbapenemases.

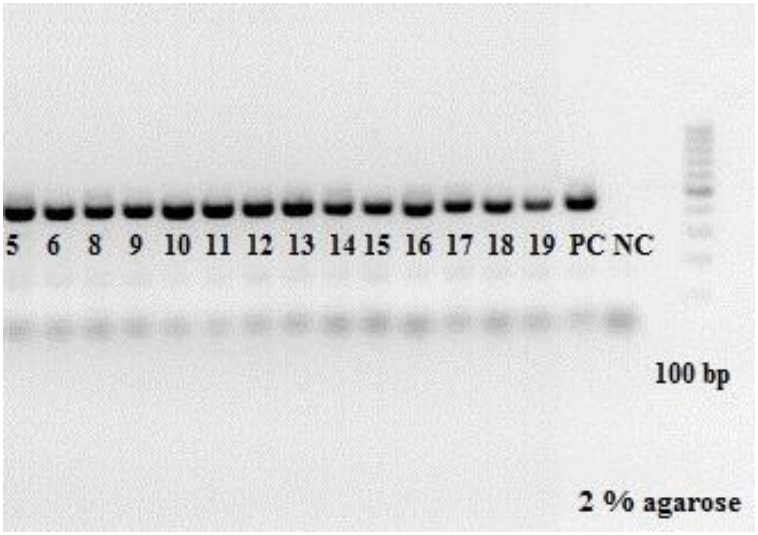
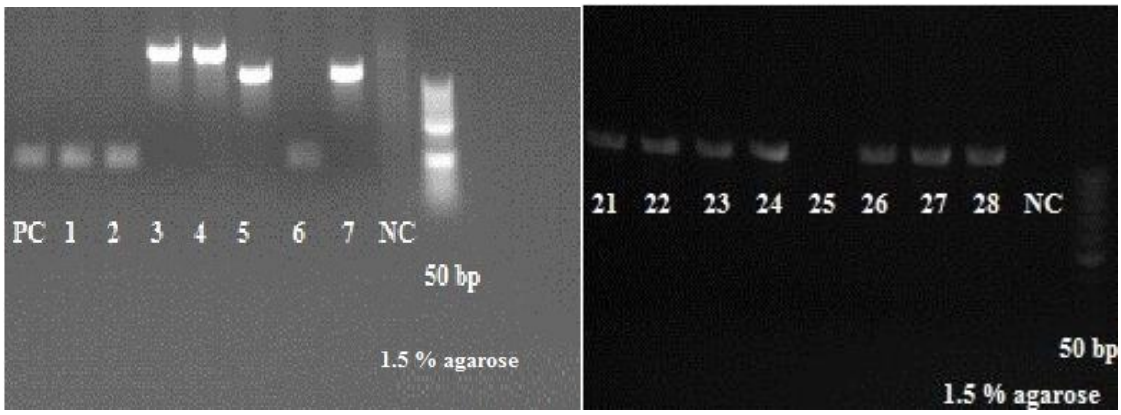


Figure 10. Gel image of multiplex PCR targeting KPC, NDM-1 and OXA-48 genes. The expected product sizes were 789, 438 and 621 bp, respectively. Only OXA-48 bands were present in gel photo. Crk17 was used as a positive control for OXA-48.

3.4 *MgrB* Gene Analysis

Of the 32 samples subjected into mgrB-Ext PCR, amplified products were detected in 31 isolates. One isolate did not yield any band, which suggested us the alteration in the binding sites of primers**.** **Figure 11** shows an agarose gel electrophoresis image of one *mgrB* gene analysis panel. A colistin susceptible clinical isolate crk0 was used as positive control. Wild type *mgrB* bands were seen in 5 isolates. Twenty-four samples had bands above 1000 bp which suggested us a presence of insertion. All of the isolates were positive for mgrB-Int PCR which means the existence of them*MgrB* gene. One of the mgrB-Int PCR analysis panel was shown in **Figure 12.**

For the further analysis of one isolate was negative by mgrB-Ext PCR amplification. larger area were screened with mgrB-EE primers and a 1507- bp product was obtained. The sequencing analysis of larger area will be sequenced soon.



A **B**

Figure 11. Agarose gel electrophoresis images of mgrB-Ext PCR panels. The expected product size 253 bp. A) crk0 was used as a positive control. crk1, crk2, crk6 had wild type band profile. However, crk3, crk4, crk5, crk7 have bands above 1000 bp. B) Crk21, crk22, crk23, crk24, crk26, crk27 and crk28 had amplified products with higher bp, also. The ones with high bands were probable IS containing samples. There is no amplified product of crk25 with mgrB-Ext primer sets.

Figure 12. Agarose gel electrophoresis images of mgrB-Int PCR panels. The expected product size was 110 bp.

The sequence analyses of the mgrB-Ext PCR products which have higher bands showed that there were four types of insertion sequences (IS) in our sample group, namely IS1 like, IS5 like, IS903 like elements and IS903B. The majority of the samples, 20 out of 32, had IS903B (62.5%). Two samples had IS5 like elements (6.3%), 2 samples had IS1 like element (6.3%) and 2 samples had IS903 like element (6.3%).

In all of the 20 isolates which their *mgrB* gene were distrupted by IS903B we observed the same short direct repeats (ACTCAGATG) at their insertion site. Similarly, two samples that containing IS5 in *mgrB* gene had the same repetitive element at their IS entry site which was TTAA. The short direct repeat of one sample containing IS1 was CAGATGCTT, however in the other sample repetitive element could not analyzed. Two

samples which had IS903 type insertion had different short direct repeats (ACTCAGATG and CAAAT).

According to integration site analysis, IS1 elements were detected at the upstream region of the gene however IS5, IS903 and IS903B elements were seen in the gene. **Figure 13** shows the insertion positions of ISs.

In the sequence analyses of 5 isolates which revealed wild type pattern after PCR, there were no insertion sequences in their *mgrB* region. However, silent mutations were detected in 3 of the 5 samples (A to C at different positions). One sample had a point mutation at the position 68 (T to A) and one sample had a frameshift mutation at the position 9 (9delA). The mutation types of the 5 wild type isolates were shown in **Table 8**.

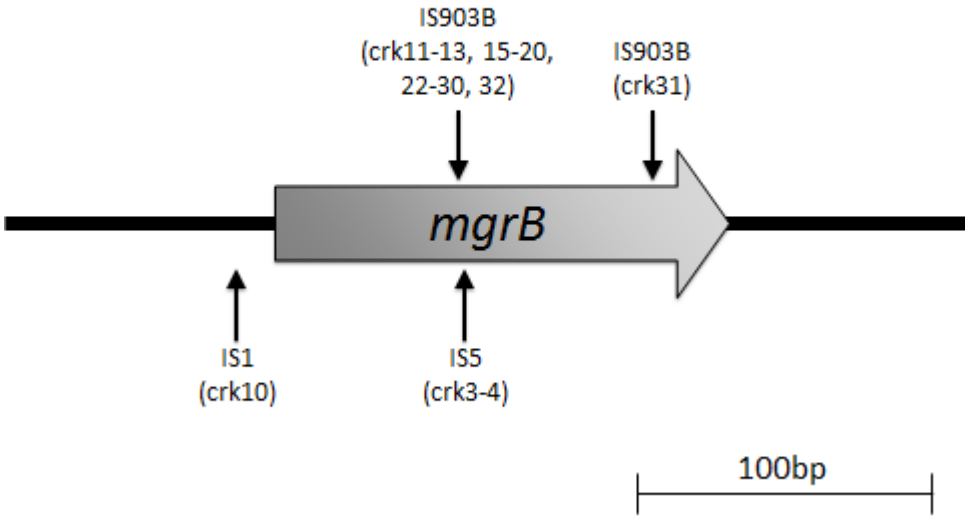


Figure 13. Insertion positions of different ISs. IS1 was found at upstream region of the *mgrB* gene, however other ISs, located into the gene.

Table 8. The Mutations in *mgrB* gene regions of 5 wild type isolates

Strain ID	Mutations
Crk1	frameshift: 9delA
Crk6	silent: 112 (A to C)
Crk8	silent: 49 (A to C)
Crk9	T68A
Crk34	silent: 112 (A to C)

3.5 *PmrK* and *PhoQ* Expression Analyses

Expression analysis revealed that there was no overexpression of *PmrK* and *PhoQ* genes. **Figure 14** and **Figure 15** show the expression data of the two genes normalized by a standard *K.pneumoniae* strain. In **Table 9** expression levels of each strain with *mgrB* status and mean standard deviation were presented.

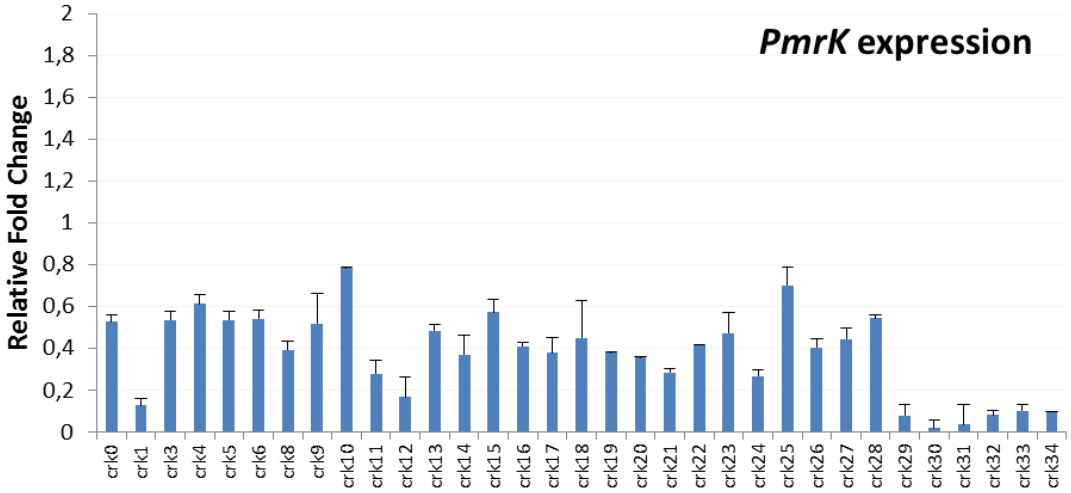


Figure 14. Relative expression levels of the, *PmrK* gene in the colistin-resistant strains compared with those in a colistin-susceptible standart calibrator strain.

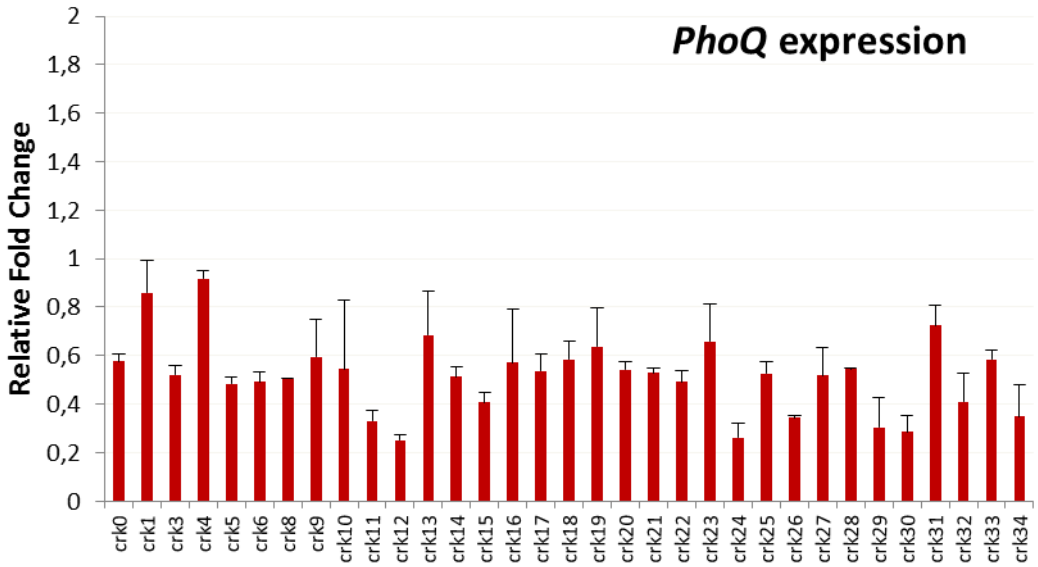


Figure 15. Relative expression levels of the, *PhoQ* gene in the colistin-resistant strains compared with those in a colistin-susceptible standart calibrator strain.

Table 9. Chromosomal *mgrB* status and expression levels of *PmrK* and *PhoQ* genes of isolates.

Isolate	Chromosomal *mgrB* status	Expression Levels (mean ± SD)	
		PmrK	***PhoQ***
crk1	Wild Type	0.13 ± 0.07	0.86 ± 0.13
crk3	Interrupted by IS5 like element	0.54 ± 0.04	0.52 ± 0.04
crk4	Interrupted by IS5 like element	0.61 ± 0.04	0.92 ± 0.03
crk5	Interrupted by IS1 like element	0.54 ± 0.04	0.48 ± 0.02
crk6	Wild Type	0.54 ± 0.04	0.49 ± 0.05
crk8	Wild Type	0.39 ± 0.04	0.50 ± 0.00
crk9	Wild Type	0.52 ± 0.13	0.59 ± 0.14
crk10	Interrupted by IS1 like element	0.78 ± 0.00	0.55 ± 0.25
crk11	Interrupted by IS903B	0.28 ± 0.06	0.33 ± 0.04
crk12	Interrupted by IS903B	0.17 ± 0.08	0.25 ± 0.02
crk13	Interrupted by IS903B	0.48 ± 0.03	0.69 ± 0.16
crk14	Interrupted by IS903B	0.37 ± 0.08	0.51 ± 0.04
crk15	Interrupted by IS903B	0.57 ± 0.06	0.41 ± 0.04
crk16	Interrupted by IS903B	0.41 ± 0.01	0.57 ± 0.19
crk17	Interrupted by IS903B	0.38 ± 0.07	0.54 ± 0.07
crk18	Interrupted by IS903B	0.45 ± 0.15	0.58 ± 0.07
crk19	Interrupted by IS903B	0.38 ± 0.00	0.64 ± 0.14
crk20	Interrupted by IS903B	0.36 ± 0.00	0.54 ± 0.03
crk21	Interrupted by IS903B	0.29 ± 0.02	0.53 ± 0.02
crk22	Interrupted by IS903B	0.42 ± 0.00	0.49 ± 0.04
crk23	Interrupted by IS903B	0.47 ± 0.09	0.66 ± 0.14
crk24	Interrupted by IS903B	0.27 ± 0.03	0.26 ± 0.06
crk25	No MgrB-ext region	0.70 ± 0.08	0.53 ± 0.04
crk26	Interrupted by IS903B	0.41 ± 0.04	0.34 ± 0.01

crk27	Interrupted by IS-903 like element	0.45 ± 0.05	0.52 ± 0.10
crk28	Interrupted by IS903B	0.55 ± 0.01	0.55 ± 0.00
crk29	Interrupted by IS903B	0.08 ± 0.04	0.30 ± 0.12
crk30	Interrupted by IS903B	0.02 ± 0.02	0.29 ± 0.06
crk31	Interrupted by IS903	0.04 ± 0.06	0.72 ± 0.08
crk32	Interrupted by IS903B	0.08 ± 0.02	0.41 ± 0.10
crk33	Interrupted by IS903B	0.10 ± 0.03	0.58 ± 0.03
crk34	Wild Type	0.10 ± 0.00	0.35 ± 0.11

3.6. rep-PCR Analysis

rep-PCR,of 32 samples revealed that in our study group, there were two main clones which contains samples of > 95 % similarity. The large clone (n=21) was composed of strains collected from İstanbul Başkent University Hospital (2 strains) and Koşuyolu Hospital (19 strains). The other clone (n= 4) was composed of the samples from Ankara Başkent University Hospital (2 strains) and İstanbul Başkent University Hospital (2 strains) (with > 95 % similarity). In the large clone, crk25 was excluded from IS identification because of low sequence quality. Out of remaining 20, 18 (90%) of the strains had IS903B type insertions and in 2 strains wild type *mgrB* was detected. The small clone of 4 samples included 2 IS5 containing strains (50%) and 2 strains carrying wild type *mgrB.* **Figure 16** presents the clonal relationship of the strains.

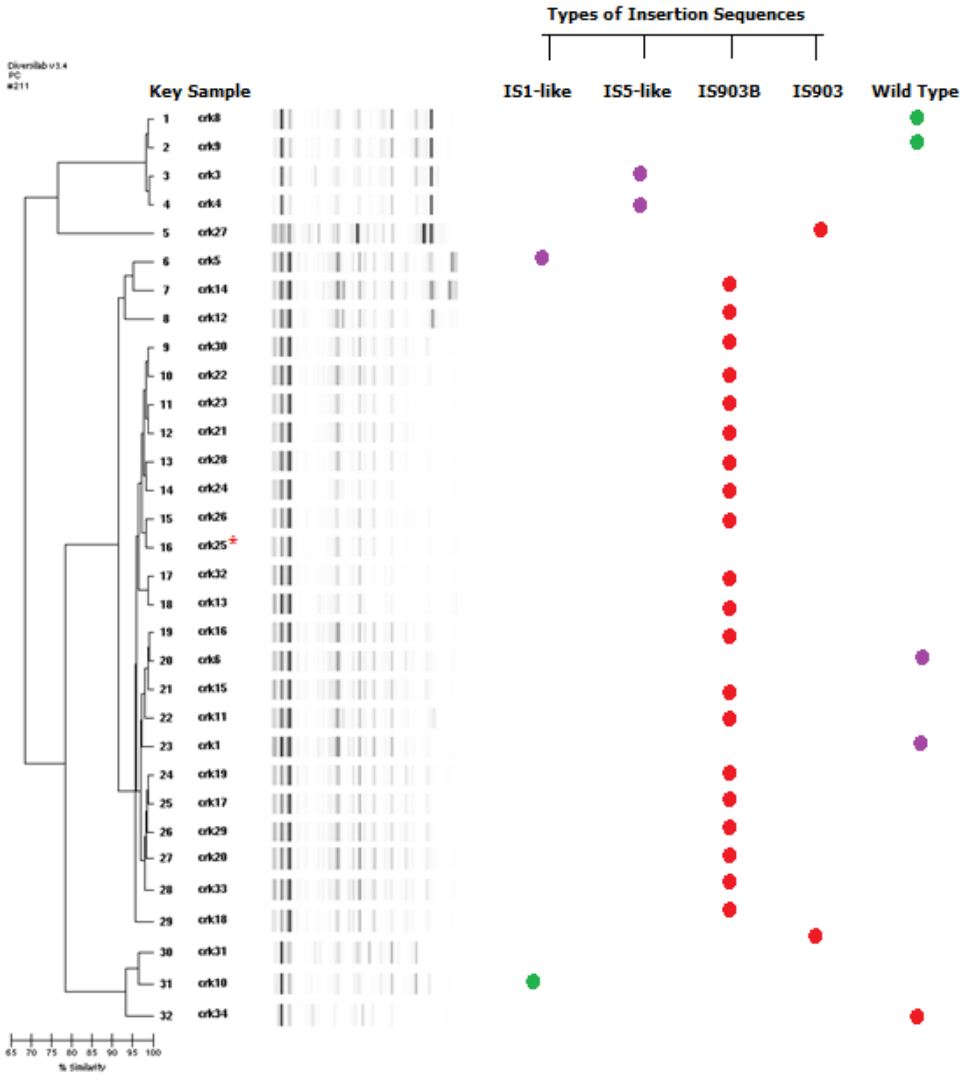


Figure 16. The dendogram of colistin resistant strains. Rep-PCR clonal analysis revealed that there were two distinct clones in our study. The colored dots represents following centers: red: Kosuyolu State Hospital, purple: İstanbul Baskent University Hospital, green: Ankara Baskent University Hospital.

*Crk 25 had no mgrB-ext region, so IS type could not be analysed.

3.7 Risk factors that may effect IS related colistin resistance

Univariate analysis of the data revealed that the mean age of patients infected with IS positive strain was 54 ± 20.1. However, the mean age of patients infected with a strain lack of IS was 80 ± 10.6.

The IS positive isolates were obtained from 95% of ICU patients and 58% of non ICU patients (P=.010). All of the patients with chronic heart failure (n=15) were infected by an IS positive strain (P=.011). The mortality rate of patients infected with an IS carrying isolate was 63% and 50% in patients with no IS in the bacterial *mgrB* gene (P=.606).

Colistin was used in 22 of 32 patients and 10 patients were treated with other antimicrobials. Any type of insertion in *mgrB* gene was found to be 84% of the isolates in both colistin received and not received groups of the patients (P=.975). Univariate analysis showed that the mean duration of colistin therapy was higher (19 days) among the patients who have IS carrying isolates than the patients with IS negative isolates (7 days) (P=.231). Out of seven patients with bacteremia, 5 of them had IS positive isolate (71%) and 2 of them had IS negative isolate (29%) (P=.285). **Table 10** shows the risk factors that may effect IS related colistin resistance.

Table 10. The risk factors for presence of insertion sequences (ISs)

Risk Factors	**IS positive strains n=26, No. (%)**	**IS negative strains n=5, No. (%)**	**P value**
Host Risk Factors			
Mean age	54	80	.019*
ICU stay	19 (95)	1 (5)	.010*
CHF	15 (100)	0	.011*
Colistin use	16 (84)	3 (16)	.604
Mean colistin treatment time (days)	19	7	.231
Bacteraemia	5 (71)	2 (29)	.285
Bacterial Factors			
MIC (mean)	16	18	.772

Chapter 4

DISCUSSION

K.pneumoniae is widely distributed pathogen among population and easily causes nosocomial infections that have a high outbreak potential. Colistin is widely used to treat infections caused by carbapenem-resistant *K. pneumoniae* although it's neurotoxic and nephrotoxic side effects. Colistin therapy became the last choice of treatment of such infections. Unfortunately, there are many studies reported that resistance against colistin has been an emerging dangerous problem throughout the world [50].

Many reports recorded high mortality rates and frequent treatment failure due to infections caused by KPC-producing bacteria however clinical and molecular data on these subjects are limited. Therefore an appropriate therapy for KPC infections was not well defined. There are some studies regarding the effects of colistin use, dosage and duration on colistin resistance which evaluated some patients' clinical data to understand the host risk factors causing colistin resistance. They mainly focused on the effect of colistin in monotherapy and its combination effect with carbapenem, tigecyline or aminoglycoside. Lee and colloquies claimed that colistin plus tigecycline therapy can prevent the fast dissemination of CRKP isolates [65]. Another study included 12 patients reported that colistin monotheraphy decreases the survival (66% mortality) [66]. Moreover, a large population based study claimed that the total treatment failure rates were not significantly different in the three most common antibiotic combinations: polymyxin plus carbapenem, polymyxin plus tigecycline, polymyxin plus aminoglycoside (30%, 29%, and 25% respectively; p=0.6) [29]. They emphasized the selection of the most effective combination of drugs for clinical success should be done urgently.

In recent years, in order to understand molecular resistance mechanisms of *K.pneumoniae* against colistin, some studies were performed but they did not give satisfactory outcomes to lighten the issue. They proved that there are two important molecular mechanisms having a major role in gaining resistance. Furthermore, there were reports claimed that an independent emergence of colistin resistantance in *Enterobacteriaceae* without colistin treatment. This results showed that there were some unknown mechanisms underlying colistin resistance [67].

In our study we investigated 32 colistin resistant strains and found four different types of insertion sequneces (IS5like, IS1, IS903, and IS903B) in 26 strains. The majority of the strains had IS903B type insertions in their *mgrB* region (21 strains). IS1 insertion sequences were observed in two samples.IS5 like element was seen in two isolates and IS903 was found in one isolate. Cannatelli and co-workers found that IS5like element in *mgrB* gene could be main reason for the increasing in colistin MICs [34]. They observed a decrease in colistin MICs after complementation studies. According to them, the position 75 could be the hot spot for the insertion of IS5 like element. In another study by the same group, IS inactivation of *mgrB* gene was detected in 22 out of 66 colistin resistant isolates and wild type *mgrB* gene was observed in 27 isolates. The IS types were IS1F like element, IS5 like element and IS*Kpn14* [45]. Moreover, Poirel et al. showed ISs in 9 of 47 colistin resistant isolates collected from France, Turkey, Colimbia and South Africa identified as IS5 like (5 strains), IS10R (2 strains), IS*Kpn13*(1 strain), IS*Kpn14* (1 strain*).* IS5 like and IS*Kpn13* entry sites were similar, at 74th base. In the same study they also showed that these resistance was turned to susceptibility pattern after complementation experiments with wild type *mgrB* gene [36]. Olaitan et al. found IS 903 (3 strains), IS5 like (1 strain), IS5D like (1 strain), and IS903 like elements (1 strain) in their study population included

32 samples from Laos, Thailand, France and Nigeria. They observed intact *MgrB* in 19 isolates. For all of the IS types they found a specific short direct repeats [62].

We analysed the mutations in five strains with wild type *mgrB* gene, and found no mutation affecting the protein function. We found different silent mutations in 4 strains. We did not observed a mutation causing stop codon and leading to a premature mgrB protein however in one isolate carrying wild type *mgrB* gene had a point mutation (T68A) causing an amino acid change from lysine to methionine and a frameshift mutation at position 9 (del9A). Cannatelli and his colloquies found nonsilent point mutations or small deletions in 13 strains out of 35. Moreover, Poirel et al. reported a stop codon as a result of mutation resulted in a truncated mgrB protein in 3 isolates. In the Olaitans' study the colisin MIC values of strains with an intact *mgrB* were between 3 and 16 mg/l. [62]. In our 4 strains with intact *mgrB, we* found high colistin MICs (between 6-32 mg/l) which suggested us another mechanism could also play a role in colistin resistance.

Another suggested mechanism of colistin resistance was overexpression of the genes controlled by *PhoQ/PhoP* and *PmrA/PmrB* regulator systems. Cannatelli et al, searched the role of overexpression of *PhoQ* and *PmrK* genes in colistin resistance. They compared 15 colistin resistant isolates with one colistin susceptible isolate. They showed that any mutation or insertional inactivation in *mgrB* gene cause overexpresssion of *PmrK and PhoQ* genes by 3-10 and 3-14 folds, respectively. The expression levels were decreased by the complementation experiments, and they conclude that *PmrK and PhoQ* systems might have a role in colistin resistance[45] For pmrA/PmrB systems, a study included 35 colistin resistant strains revealed that mutations in *pmrB* gene resulted in 40 fold increase in the expression of *PmrK* gene and this increase is responsible for LPS layer modification [39]. In our 32 resistant isolates we tested the expression levels of *PmrK* and *PhoQ*. In our study, there was no overexpression of both of the genes, so our data did not support their findings.

Our results showed that an insertional inactivation of the *mgrB* gene might be the major mechanism rather than *PmrK* and *PhoQ* over expression.

In the clonal analysis two distinct colistin resistant *K.pneumoniae* clones were seen in our study population. In the big clone the presence of 3 different *MgrB* statuses (IS903, IS903B and wild type) suggested us there could be a horizontal gene transfer between strains. Also the existence of the same IS types in different clones, supported the study by Poirel et al. which reported clonally unrelated strains have the same genetic events in their *mgrB* gene.[36]

The high antibiotic resistance of our strains to meropenem and ceftriaxone and ciprofloxacin which were above 50% demonstrates the significance of the threat. In colistin resistant Klebsiella spp. collected from different regions of the world, Olaitan et al. reported lower resistance rates than our study. We also found that 38% of our strains had the multidrug resistance (MDR) profile which is defined as being resistant to at least one agent in three or more different classes of antibiotics. All of the isolates were positive for OXA-48. These findings demonstrate the severity antibiotic resistance problem in Turkey.

As our knowledge, there was no scientific report about the effects of host risk factors on molecular colistin resistance mechanisms. In this study we observed inactivation of *mgrB* gene with transposons was the major mechanism for colistin resistance and we investigated various host risk factors which may cause transposon inactivation of that gene. According to univariate analyses, ICU stay increases the risk of infection with *K.pneumoniae* which have IS in their *mgrB* genes. The mean age of the patients infected by a IS positive strain was relatively lower than patients infected with non-IS isolate (P=.019). We found that colistin usage did not affect IS related colistin resistance (P=.604). But the duration of colistin therapy might be associated with acquiring this type colistin resistance (P=.231). We found that IS related colistin resistance was seen more commonly in patients treated with colistin for 19 days or more (P= .231). The limitation of this study is low sample size.

We think that in larger population the difference in the duration of therapy between two groups will be more significant.

In conclusion, we report the first findings on the molecular and clinical epidemiology of colistin resistance among *K.pneumoniae*. The results of the study show that disruption of *mgrB* gene by transposons is the key mechanism for colistin resistance. Long term stay in intensive care unit, and relatively younger age were major host risk factors that increase the risk of infection by a *K.pneumoniae* strain carrying IS in its *mgrB* gene. Prolonged colistin therapy might have an effect on IS related colistin resistance. Demonstration of the horizontal gene transfer between colistin resistant strains highlights the emergence of colistin resistance as the public health problem of the world.

BIBLIOGRAPHY

1. Disease, C. Prevention of hospital-acquired infections World Health Organization. (2002).

2. Inweregbu, K. Nosocomial infections. *Contin. Educ. Anaesthesia, Crit. Care Pain* **5,** 14–17 (2005).

3. Boucher, H. W. *et al.* Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48,** 1–12 (2009).

4. Malhotra, S., Sharma, S. & Hans, C. Prevalence of Hospital Acquired Infections in a tertiary care hospital in India. **1,** 91–94 (2014).

5. Grossien Mark. Healthcare Associated Infections Cost Europe €5.5 Billion Per Year. (2009) retrieved from http://medtechviews.eu

6. Gray, J. & Omar, N. Nosocomial infections in neonatal intensive care units in developed and developing countries: how can we narrow the gap? *J. Hosp. Infect.* **83,** 193–5 (2013).

7. Alp, E., Coruh, A., Gunay, G. K., Yontar, Y. & Doganay, M. Risk factors for nosocomial infection and mortality in burn patients: 10 years of experience at a university hospital. *J. Burn Care Res.* **33,** 379–85 (2011).

8. Gaynes, R. & Edwards, J. R. Overview of nosocomial infections caused by gram-negative bacilli. *Clin. Infect. Dis.* **41,** 848–54 (2005).

9. Azcuna, J. M. Hospital acquired infections in the multi-resistant bacteria era. *RIPER PDIC Bull.* **2,** 7–12 (2011).

10. CDC, U. S. D. of H. and H. S. Antibiotic resistance threats. (2013).

11. Laxminarayan, R. *et al.* in *Disease Control Priorities in Developing Countries. 2nd edition.* 1031–1051 (World Bank, 2006).

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

13. Yenilmez, E. The Current Definitions of Health-Care Associated Infections in Intensive Care Units. *J. Clin. Anal. Med.* **6,** 401–404 (2015).

14. Ergönül, Ö. ve ark. Sağlık Bakımıyla ilişkili Gram Negatif Bakteremilerde Fataliteyi Belirleyen Faktörlerin Araştırılması, Ulusal Sağlık Bakımıyla İlişkili İnfeksiyonlar Simpozyumu, İstanbul, (2014).

15. Brisse, S. *et al.* Virulent clones of Klebsiella pneumoniae: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS One* **4,** e4982 (2009).

16. Meyer, M., Dimroth, P. & Bott, M. Catabolite Repression of the Citrate Fermentation Genes in Klebsiella pneumoniae: Evidence for Involvement of the Cyclic AMP Receptor Protein. *J. Bacteriol.* **183,** 5248–5256 (2001).

17. Roxana B. Hughes. No Title. (2010). at <http://www.microbelibrary.org/library/2-associated-figure-resource/1798-klebsiella-pneumoniae-capsule-stain-enlarged-view>

18. Tärnberg, M. Extended-spectrum beta-lactamase producing Enterobacteriaceae : aspects on detection , epidemiology and multi-drug resistance. 13–18 (Linköping University, 2012).

19. Gupta, A., Ampofo, K., Rubenstein, D. & Saiman, L. Extended spectrum beta lactamase-producing Klebsiella pneumoniae infections: a review of the literature. *J. Perinatol.* **23,** 439–43 (2003).

20. Cortés, G., Álvarez, D., Saus, C. & Albertı, S. Role of Lung Epithelial Cells in Defense against Klebsiella pneumoniae Pneumonia. **70,** 1075–1080 (2002).

21. Decré, D. *et al.* Emerging severe and fatal infections due to Klebsiella pneumoniae in two university hospitals in France. *J. Clin. Microbiol.* **49,** 3012–4 (2011).

22. Tzouvelekis, L. S., Markogiannakis, a, Psichogiou, M., Tassios, P. T. & Daikos, G. L. Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: an evolving crisis of global dimensions. *Clin. Microbiol. Rev.* **25,** 682–707 (2012).

23. Highsmith Anita K., J. W. R. Klebsiella pneumoniae: Selected Virulence Factors That Contribute to Pathogenicity. *Infect. Control* **6,** 75–77 (1985).

24. The cell wall structure. at <http://classroom.sdmesa.edu/eschmid/Lecture3-Microbio.htm >

25. Ullmann, U. Klebsiella spp . as Nosocomial Pathogens : Epidemiology , Taxonomy , Typing Methods , and Pathogenicity Factors. **11,** 589–603 (1998).

26. Greenwood, D., Slack, R.C.B., & Peutherer, J. . in *Medical microbiology: A guide to microbial infections: Pathogeneisis, immunity, laboratory diagnosis and control* (ON: Churchill Livingstone, 2002).

27. Temkin, E., Adler, A., Lerner, A. & Carmeli, Y. Carbapenem-resistant Enterobacteriaceae: biology, epidemiology, and management. *Ann. N. Y. Acad. Sci.* **1323,** 22–42 (2014).

28. Nordmann, P., Cuzon, G. & Naas, T. The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. *Lancet. Infect. Dis.* **9,** 228–36 (2009).

29. Lee, G. C. & Burgess, D. S. Treatment of Klebsiella pneumoniae carbapenemase (KPC) infections: a review of published case series and case reports. *Ann. Clin. Microbiol. Antimicrob.* **11,** 32 (2012).

30. Dortet, L., Cuzon, G. & Nordmann, P. Dissemination of carbapenemase-producing Enterobacteriaceae in France, 2012. *J. Antimicrob. Chemother.* **69,** 623–7 (2014).

31. Carrer, a. *et al.* Spread of OXA-48-Encoding Plasmid in Turkey and Beyond. *Antimicrob. Agents Chemother.* **54,** 1369–1373 (2010).

32. Zeka, a. N. *et al.* GES-type and OXA-23 carbapenemase-producing Acinetobacter baumannii in Turkey. *J. Antimicrob. Chemother.* **69,** 1145–1146 (2013).

33. Poirel, L. *et al.* NDM-1-Producing Klebsiella pneumoniae Now in Turkey. *Antimicrob. Agents Chemother.* **56,** 2784–2785 (2012).

34. Cannatelli, A. *et al.* In vivo emergence of colistin resistance in Klebsiella pneumoniae producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. *Antimicrob. Agents Chemother.* **57,** 5521–5526 (2013).

35. Broberg, C. a, Palacios, M. & Miller, V. L. Klebsiella: a long way to go towards understanding this enigmatic jet-setter. *F1000Prime Rep.* **6,** 64 (2014).

36. Poirel, L. *et al.* The mgrB gene as a key target for acquired resistance to colistin in Klebsiella pneumoniae. *J. Antimicrob. Chemother.* (2014). doi:10.1093/jac/dku323

37. Falagas, M. E. & Kasiakou, S. K. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin. Infect. Dis.* **40,** 1333–41 (2005).

38. Raveh, D. *et al.* Colistin : An Antimicrobial for the 21st Century ? Syndrome Associated with Abacavir Therapy of Enterococcus faecalis Prosthetic Valve Endocarditis with Linezolid. **35,** 901–902 (2002).

39. Jayol, A. *et al.* Resistance to colistin associated with a single amino acid change in protein PmrB among Klebsiella pneumoniae isolates of worldwide origin. *Antimicrob. Agents Chemother.* **58,** 4762–4766 (2014).

40. Dalfino, L. *et al.* High-dose, extended-interval colistin administration in critically ill patients: is this the right dosing strategy? A preliminary study. *Clin. Infect. Dis.* **54,** 1720–6 (2012).

41. Petrosillo, N. *et al.* Clinical experience of colistin-glycopeptide combination in critically ill patients infected with Gram-negative bacteria. *Antimicrob. Agents Chemother.* **58,** 851–8 (2014).

42. Halaby, T., Al Naiemi, N., Kluytmans, J., van der Palen, J. & Vandenbroucke-Grauls, C. M. J. E. Emergence of colistin resistance in Enterobacteriaceae after the introduction of selective digestive tract decontamination in an intensive care unit. *Antimicrob. Agents Chemother.* **57,** 3224–9 (2013).

43. Kontopidou, F. *et al.* Colonization and infection by colistin-resistant Gram-negative bacteria in a cohort of critically ill patients. *Clin. Microbiol. Infect.* **17,** E9–E11 (2011).

44. Olaitan, A. O., Morand, S. & Rolain, J.-M. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front. Microbiol.* **5,** 1–18 (2014).

45. Cannatelli, A. *et al.* MgrB inactivation is a common mechanism of colistin resistance in KPC carbapenemase-producing Klebsiella pneumoniae of clinical origin. *Antimicrob. Agents Chemother.* **58,** 5696–703 (2014).

46. Capone, A. & Giannella, M. High rate of colistin resistance among patients with carbapenem-resistant Klebsiella pneumoniae infection accounts for an excess of mortality. *Clin. Microbiol. Infect.* **9**, *23-30* (2013).

47. 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).

48. Hirsch, E. B. & Tam, V. H. Detection and treatment options for Klebsiella pneumoniae carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *J. Antimicrob. Chemother.* **65,** 1119–25 (2010).

49. Tumbarello, M. *et al.* Predictors of mortality in bloodstream infections caused by Klebsiella pneumoniae carbapenemase-producing K. pneumoniae: importance of combination therapy. *Clin. Infect. Dis.* **55,** 943–50 (2012).

50. Marchaim, D. *et al.* Outbreak of colistin-resistant, carbapenem-resistant Klebsiella pneumoniae in metropolitan Detroit, Michigan. *Antimicrob. Agents Chemother.* **55,** 593–9 (2011).

51. 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).

52. Nikaido, H. Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiol. Mol. Biol. Rev.* **67,** 593–656 (2003).

53. Cheng, H.-Y., Chen, Y.-F. & Peng, H.-L. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in Klebsiella pneumoniae CG43. *J. Biomed. Sci.* **17,** 60 (2010).

54. Barrow, K. & Kwon, D. H. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **53,** 5150–4 (2009).

55. Kox, L. F. F., Wo, M. M. S. M. & Groisman, E. A. A small protein that mediates the activation of a two-component system by another two-component system. **19,** 1861–1872 (2000).

56. Kato, A., Latifi, T. & Groisman, E. A. Closing the loop : The PmrA ¯PmrB two-component system negatively controls expression of its posttranscriptional activator PmrD. **100,** 4706–4711 (2003).

57. Winfield, M. D. & Groisman, E. A. Phenotypic differences between Salmonella and Escherichia coli resulting from the disparate regulation of homologous genes. **725333,** (2004).

58. Groisman, E. A. Minireview: The Pleiotropic Two-Component Regulatory System PhoP-PhoQ. *Journal of Bacteriology*, **183,** 1835–1842 (2001).

59. Lippa, A. M. & Goulian, M. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet.* **5,** e1000788 (2009).

60. López-Camacho, E. *et al.* Genomic analysis of the emergence and evolution of multidrug resistance during a Klebsiella pneumoniae outbreak including carbapenem and colistin resistance. *J. Antimicrob. Chemother.* **69,** 632–6 (2014).

61. Gaibani, P. *et al.* In vitro activity and post-antibiotic effects of colistin in combination with other antimicrobials against colistin-resistant KPC-producing Klebsiella pneumoniae bloodstream isolates. *J. Antimicrob. Chemother.* **69,** 1856–65 (2014).

62. Olaitan, A. O. *et al.* Worldwide emergence of colistin resistance in Klebsiella pneumoniae from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator mgrB: an epidemiological and molecular study. *Int. J. Antimicrob. Agents* **44,** 500–7 (2014).

63. Clinical and Laboratory Standards Institute . Performance standards for Anti-microbial Susceptibility Testing : 20th Informational Supplement. Wayne, PA : CLSI (2010).

64. Poirel, L., Walsh, T. R., Cuvillier, V. & Nordmann, P. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn. Microbiol. Infect. Dis.* **70,** 119–23 (2011).

65. Lee, J., Patel, G., Huprikar, S., Calfee, D. P. & Jenkins, S. G. Decreased susceptibility to polymyxin B during treatment for carbapenem-resistant Klebsiella pneumoniae infection. *J. Clin. Microbiol.* **47,** 1611–2 (2009).

66. Qureshi, Z. a *et al.* Treatment outcome of bacteremia due to KPC-producing Klebsiella pneumoniae: superiority of combination antimicrobial regimens. *Antimicrob. Agents Chemother.* **56,** 2108–13 (2012).

67. Chen, S. *et al.* Independent emergence of colistin-resistant Enterobacteriaceae clinical isolates without colistin treatment. *J. Clin. Microbiol.* **49,** 4022–3 (2011).

VITA

Pelin İspir was born in Ankara, Turkey on September, 10, 1990. She graduated from Yavuz Sultan Selim Anatolian High School in Ankara in 2008. She received her Bachelor of Science Degree with a major in Biology from Middle East Technical University. She has done her M. Sc studies together with research and teaching assistantship at Koç University from September 2013 to June 2015.