Molecular Epidemiology of Carbapenem Resistant *Klebsiella pneumoniae* in Bloodstream Infections

by

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ABSTRACT

Carbapenem resistant *Klebsiella pneumoniae* (CRKP) has become a clinically important pathogen causing worldwide nosocomial infections that are associated with high rates of mortality. High risk clones of CRKP carrying KPC, NDM and OXA-48-like carbapenemases have spread worldwide. Resistance to carbapenem has limited the effective and safe treatment options, thus causing increased patient fatality. In this study, our aim was to describe molecular epidemiology of CRKP bacteremia in Turkey and to evaluate the effect of carbapenemase producing high risk clones on mortality.

A total of 254 CRKP bacteremia isolates from 13 tertiary care centers in Turkey were collected between June 2018 and June 2019. The 30-day mortality of the patients was recorded. Meropenem, colistin, and ceftazidime/avibactam minimum inhibitory concentrations were determined by meropenem Etest and broth microdilution methods. Carbapenemase genes ($bla_{OXA-48-like}$, bla_{KPC-2}, bla_{NDM-1}) were detected by multiplex PCR. For genotyping of the isolates pulsed field gel electrophoresis and multilocus sequence typing were performed. All analyses were performed using STATA software version 16.0.

The ST2096 constituted the largest clonal type with 62 isolates (30%) followed by ST101 with 36 isolates (18%) and ST14 with 27 isolates (13%) in the CRKP group. Fifty-one of 254 isolates were detected as meropenem susceptible and classified as carbapenem susceptible *Klebsiella pneumoniae*. The OXA-48-like carbapenemases comprised the largest carbapenemase group with 171 isolates (91%), NDM-1 was detected in 40 isolates (21%). OXA-48-like carbapenemases were mainly carried on ST2096 in 61 isolates and on ST101 in 35 isolates. The number of OXA-48-like and NDM-1 co-producer was 30 (16%) and 19 of them (63%) belonged to the ST14 clone. Overall colistin susceptibility was found as 38%. The ST101 and ST2096 clonal types had high colistin resistance rates with 83% and 81%, respectively. Overall ceftazidime-avibactam was the most active antibiotic with 84% susceptibility rate but the isolates belonging to ST14 had the lowest susceptibility rate (22%). The 30-day all-cause mortality was 40% for all patients. Mortality rates in infections with the ST2096, ST14 and ST101 clonal groups were 50%, 53%, and 41%, respectively. ST2096 (P=0.02) and ST14 (P=0.002) clonal types were found to be associated with high mortality.

In conclusion, the emerging CRKP ST2096 clone possessing OXA-48-like is now the predominating clonal type in Turkish hospitals. Epidemiology of carbapenemases is shifting towards MBL and non-MBL co-producers. High colistin resistance and mortality rates in clones of CRKP ST14 and ST2096 indicates successful adaptation and severe disease production capacity of these clones. High ceftazidime/avibactam resistance in MBL producer ST14 clone demonstrates an urgent need for new antibiotics that are active not only against OXA-48-like producers but also against MBL producers.

ÖZETÇE

Karbapenem dirençli *Klebsiella pneumoniae* (KDKP), yüksek mortalite oranları ile ilişkili dünya çapında nozokomiyal enfeksiyonlara neden olan klinik olarak önemli bir patojen haline gelmiştir. KPC, NDM ve OXA-48 benzeri karbapenmeazlar üreten KDKP'nın yüksek riskli klonları dünya çapında yayılmıştır. Karbapeneme direnç, etkili ve güvenli tedavi seçeneklerini sınırlandırarak, hasta ölümlerinin artmasına neden olmuştur. Bu çalışmada amacımız, Türkiye'deki KDKP bakteriyemisinin moleküler epidemiyolojisinin tanımlanması ve karbapenemaz üreten yüksek riskli klonların mortaliteye etkisinin değerlendirilmesidir.

Bu amaç doğrultusunda, 2018 Haziran ve 2019 Haziran tarihleri arasında Türkiye'deki 13 üçüncü basamak sağlık merkezinden toplam 254 karbapenem dirençli *Klebsiella pneumoniae* bakteremi izolatı toplanmıştır. Hastaların 30 günlük mortalite bilgileri kayıt altına alınmıştır. Meropenem, kolistin ve seftazidim/avibaktam minimum inhibitör konsantrasyonları meropenem Etest ve sıvı mikrodilüsyon yötemleri ile belirlenmiştir. Karbapenemaz genleri ($bla_{OXA\text{-}48\text{-}benzeri}$, $bla_{KPC\text{-}2}$, $bla_{NDM\text{-}1}$) multipleks PZR ile tespit edilmiştir. İzolatların genotiplendirilmesi için değişken alanlu jel elektroforezi ve multilokus sekans tiplemesi yapılmıştır.Tüm analizler STATA yazılım versiyonu 16.0 kullanılarak yapılmıştır.

KDKP grubunda ST2096 62 izolatla (30%) en büyük klonal tipi oluştururken, onu 36 izolatla (%18) ST101 ve 27 izolatla (%13) ST14 izlemektedir. 254 izolattan 51'inin meropeneme duyarlı olduğu tespit edilmiş ve bu izolatlar karbapenem duyarlı *Klebsiella pneumoniae* olarak sınıflandırılmıştır. OXA-48 benzeri karbapenemazlar 171 izolatla (%91) en büyük karbapenemaz grubunu oluştururken, 40 izolatta NDM-1 (%21) tespit edilmiştir. OXA-48 benzeri karbapenemazlar 61 izolatta ST2096 üzerinde ve 35 izolatta ST101 üzerinde taşınmaktadır. OXA-48 benzeri ve NDM-1 ortak yapımcı sayısı 30'dur (16%) ve bunların 19'u ST14 klonuna aittir (%63). Genel kolistin duyarlılığı %38 olarak bulunmuştur. ST101 ve ST2096 klonal tipleri, sırasıyla %83 ve %81 ile yüksek kolistin direnç oranlarına sahiptir. Genel olarak seftazidim-avibaktam %84 duyarlılık oranı ile en aktif antibiyotik olmakla birlikte ST14 klonuna ait izolatlar en düşük duyarlılık oranına sahiptir (%22). Otuz günlük tüm nedenlere bağlı mortalite tüm hastalar için %40'tır. ST2096, ST14 ve ST101 klonal grupları ile enfeksiyonlarda ölüm oranları sırasıyla %50, %53 ve %41'dir. ST2096 ($P = 0.02$) ve ST14 ($P = 0.002$) klonal tipleri mortalite ile ilişkili bulunmuştur.

Sonuç olarak, ST2096, Türkiye'deki hastanelerde OXA-48 benzeri karbapenemaz üreten ve yeni ortaya çıkan baskın klonal tip olarak karşımıza çıkmaktadır. Karbapenemazların epidemiyolojisi, MBL/MBL dışı ortak üreticilere doğru kaymaktadır. CDKP'nın ST14 ve ST2096 klonlarında yüksek kolistin direnci ve ölüm oranları, bu klonların başarılı adaptasyonunu ve ciddi hastalık üretim kapasitesini göstermektedir. MBL üreticisi ST14 klonundaki yüksek seftazidim/avibaktam direnci, sadece OXA-48-benzeri üreticilerine karşı değil aynı zamanda MBL üreticilerine karşı da aktif olan yeni antibiyotiklere acil olarak ihtiyaç duyulduğunu göstermektedir.

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ABBREVIATIONS

BMD	Broth Microdilution Method
BSI	Bloodstream infections
CDC	Centers for Disease Control and Prevention
CRE	Carbapenem resistant *Enterobacteriaceae*
CRKP	Carbapenem-resistant *Klebsiella pneumoniae*
ESBL	Extended Spectrum Beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ICU	Intensive Care Units
KPC	*Klebsiella pneumoniae* carbapenemases
MDR	Multiple Drug Resistance
MH	Mueller Hinton II Broth
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
NDM	New Delhi metallo-β-lactamase
OMP	Outer membrane protein
OXA	Oxacillinase
PBP	Penicillin-binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
SBL	Serine β-lactamases
ST	Sequence Type
TSA	Tryptic Soy Agar
USA	The United States of America
VIM	Verona integron-encoded metallo-β-lactamase
WGS	Whole-genome Sequencing
WHO	World Health Organization

Chapter 1:
INTRODUCTION

Klebsiella pneumoniae which is opportunistic gram-negative bacteria is found as part of normal intestinal microflora. *K.pneumoniae* may cause severe infections when bacteria disseminate into circulation or other tissues and it is among the most common causes of nosocomial infections of respiratory tract, urinary tract, and bloodstream infection [1-3]. *K.pneumoniae* has emerged as an urgent threat since it is associated with high rates of antibiotic resistance and fatality, particularly in immunocompromised individuals [2, 3].

Carbapenem-resistant *K.pneumoniae* (CRKP) has emerged as a worldwide threat over the past two decades. CRKP is endemic in many countries because of rapidly spread of carbapenem-hydrolyzing beta-lactamases called as carbapenemases [4, 5]. CRKP has been listed by the Centers for Disease Control and Prevention (CDC) and The World Health Organization (WHO) as one of the most urgent antibiotic resistant bacteria for which new antibiotics are urgently needed [6]. CRKP is responsible for the largest proportion of the increase in deaths caused by antibiotic resistant bacteria in Europe [7]. Resistance to multiple antibiotics of beta-lactams limits efficacious and safe treatment options. Mortality rates can be as high as 40 to 50%, because of the difficulty of effective treatment [8]. The bloodstream infections due to CRKP have a mortality rate of 40% to 70%, while this rate is 20% to 30% for carbapenem susceptible infections [9].

1.1 *Klebsiella pneumoniae*

1.1.1 Characteristics and Identification of *Klebsiella pneumoniae*

Klebsiella pneumoniae which is a member of the Klebsiella genus of Enterobacteriaceae family is a Gram-negative, non-motile, rod shaped, encapsulated, lactose fermenting, facultative anaerobic, catalase positive, oxidase negative bacterium [1, 10]. *K.pneumoniae* colonies are large shiny and dark pink colored due to the lactose fermentation on MacConkey agar plate [10]. *K.pneumoniae* has a pronounced capsule that is responsible for the mucoid appearance of isolated colonies. Capsular polysaccharide which is known as K antigen protects the bacteria from phagocytosis by resisting complement-mediated killing thus causing enhanced virulence of the bacteria in

vivo [10, 11]. *K.pneumoniae* has been classified into serotypes. Serotyping is based on distinct variations of lipopolysaccharide (O antigen) and capsular polysaccharide (K antigen). The number of serotypes has been estimated to be eight for O-antigens and 77 for K-antigens to date [12]. Figure 1.1 displays the light microscope image of Gram staining and characteristic mucoid phenotype of *K.pneumoniae*.

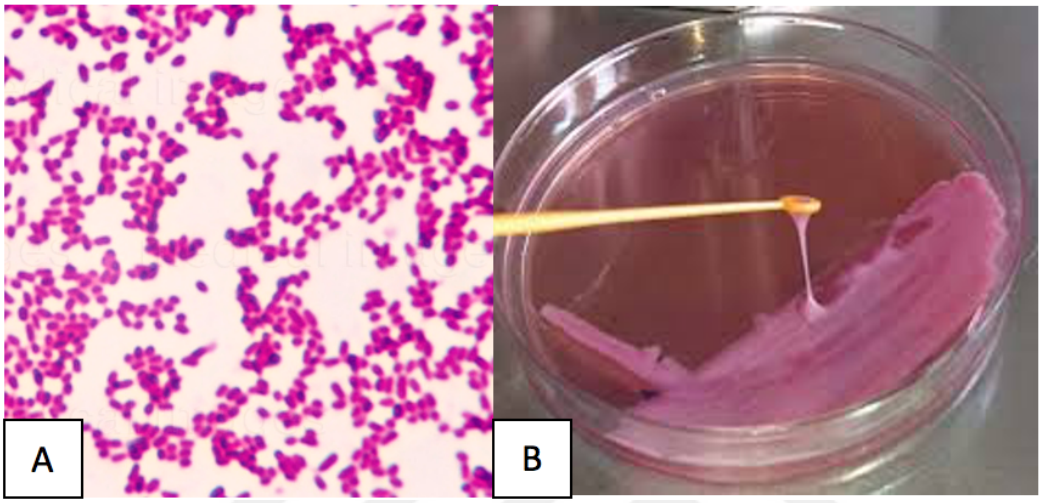

Figure 1.1: **A.** light microscope image of Gram staining of *K.pneumoniae*. **B.** characteristic mucoid-capsular phenotype of *K.pneumoniae* on MacConkey agar plate.

K.pneumoniae has become a clinically important pathogen, causing worldwide nosocomial infections with high rates of antibiotic resistance and mortality [13]. Unfortunately, use of limited routine culture and conventional biochemical tests for distinguishing the species leads to incorrect identification of *K.pneumoniae*. In the last years, one-step multiplex PCR (Polymerase Chain Reaction) methods were proposed in the clinical routine, but they were insufficient to distinguish all phylogroups of *Klebsiella* [14]. Whole-genome sequencing (WGS) or sequencing of species-specific genes are reliable methods for identification of different members of *K.pneumoniae* but these techniques are not available in many routine laboratories due to speed, cost and throughput limitations. Recently, MALDI-TOF MS (Matrix assisted laser desorption ionization time of flight mass spectrometry) as a reliable, cost-effective and fast identification method has been suggested for identification of *K. pneumoniae* [15].

1.1.2 History of Antibiotic Resistance in *Klebsiella pneumoniae*

In 1882, *K.pneumoniae* was first isolated from lungs of a patient by Carl Friedlander [2]. The penicillinase enzyme was discovered in 1940 before widespread use of penicillin through utilization of mass production techniques in 1943 [16]. In the 1960s penicillin resistant *K. pneumoniae* was reported. *K.pneumoniae* is intrinsically resistant to penicillin because of the possessing of bla_{SHV-1} and bla_{TEM-1} β-lactamase genes in its chromosome. Two decades later, bla_{SHV-2} was identified in *K. pneumoniae* isolated from a patient in intensive care unit. The bla_{SHV-2} gene enables *K. pneumoniae* isolates to be resistant against β-lactam group antibiotics, including third generation cephalosporins and monobactams [17, 18]. The late 1980s and early 1990s, plasmid-mediated AmpC genes emerged through incorporation of β-lactamase genes encoded on chromosomes onto transferable plasmids [19]. During the 1990–2000s, *K. pneumoniae* has become the major ESBL-carrying pathogen with the emergence of new plasmid-mediated ESBLs. In the 2000s, acquisition of transposable elements and plasmids encoding blaCTX-M-type ESBL caused hospital outbreaks that led to be predominant CTX-M-producer strains [20]. The endemic occurrence of ESBL-producer *K. pneumoniae* worldwide has led to exponential evolution of new ESBL-types and alleles, acquired by horizontal transfer of ESBL-encoding plasmids and transposons. Until 2011, more than 700 different ESBLs have been described [21]. The muti-drug resistance caused by ESBL-producing *K. pneumoniae* strains has led to extensive use of carbapenem, thus causing carbapenem resistance through evolution of plasmid-mediated carbapenemases [22]. Carbapenemase–producing *K. pneumoniae* strains have become major carbapenem resistant *Enterobacteriaceae* (CRE) to have spread worldwide because of their increasing frequency in antibiotic resistance [23].

1.2 Carbapenems

Carbapenems are a member of β-lactam group antibiotics that possess a β-lactam ring. Carbapenems that have a unique molecular structure with having a five-membered ring, which serves noticeable resistance against the most β-lactamases, including extended spectrum β-lactamases (ESBLs) [24]. Figure 1.2. displays the backbone structure of carbapenem.

Figure 1.2: Carbapenem backbone structure.

First carbapenem was discovered from *Streptomyces cattleya* as thienamycin in 1976. N-formimidoyl derivative, called imipenem, was semi synthetically produced since the unstable nature of thienamycin in water limited its clinical use [25]. The administration of imipenem with cilastatin inhibits imipenem renal degradation with having an antagonist effect for renal tubular dipeptidase enzyme [26]. Ertapenem, meropenem, doripenem and imipenem/cilastatin that are the members of carbapenems are utilized for treatment of severe infections against both gram-negative and gram-positive bacteria [27, 28]. Meropenem is less effective against Gram-positive bacteria (especially Enterococcus) compared to imipenem. Meropenem are used especially in infections originated by Gram-negative bacteria except *Acinetobacter baumannii* since the pyrrolidinyl substituent at the 2-position of meropenem's side chain improves activity against Gram-negative bacteria and stability toward tubular dipeptidase enzyme [24, 29]. Doripenem is more active against some resistant *Acinetobacter baumannii* and *Pseudomonas* strains [28, 30]. Ertapenem has a more limited spectrum and has less activity against *Enterococcus, Acinetobacter* species and *Pseudomonas aeruginosa*, and compared to imipenem, doripenem and meropenem [31]. However, since ertapenem has long half-life, it is used in the empiric treatment of community-acquired intra-abdominal infections caused by *Enterobacteriaceae* [24].

Carbapenems bind to penicillin-binding proteins (PBPs) and exhibit bactericidal activity. PBPs have an important role in bacterial cell wall synthesis [24]. Bacterial cell wall is

composed of a peptidoglycan layer. Transpeptidase enzymes called PBPs catalyzes the synthesis of peptidoglycan which is the last transpeptidation step [32]. Carbapenems are not diffusible through the bacterial cell wall, thus they have to enter Gram-negative bacteria through outer membrane porin proteins (OMPs)[33]. After translocation of carbapenem to periplasmic space, carbapenems bind permanently to the active site of the PBPs. The acylated D-alanyl-D-alanine which is the terminal amino acid residues of the peptidoglycan is structurally similar to carbapenem. This similarity facilitates permanently acylation of PBPs, thus inhibits the transpeptidation of the peptidoglycan layer[32, 34]. After the inhibition of cell wall biosynthesis, existing peptidoglycan is digested by autolytic hydrolases. Autolysins that are a group of bacterial surface enzymes create weak spots in the cell wall through which the cell membrane extrudes. Cell membrane is not able to keep the hypertonic cell from rupturing by osmotic shock, thus leading to bacterial cell death [28, 35]. In Figure 1.3. the schematic representation of mechanisms of action of carbapenems are illustrated.

An important factor in the effectiveness of carbapenems is their capability to bind to multiple different PBPs [36]. Imipenem binds most strongly to PBP2, followed by PBP1a and PBP1b but it has weak affinity for PBP3 [37]. Meropenem and ertapenem bind preferentially to PBP2, followed by PBP3, but also have strong affinities for PBP1a and PBP1b ([29, 37]. The affinity of doripenem for PBP is species specific. Doripenem binds strongly to PBP3 in *Pseudomonas aeruginosa*, to PBPs 1, 2 and 4 in *Staphylococcus aureus*, and PBP2 in *Escherichia coli* [38]. Carbapenems bind preferentially to PBPs 1a, 1b and 2 rather than PBP3 with greatest affinity in Gram-negative bacteria [29].

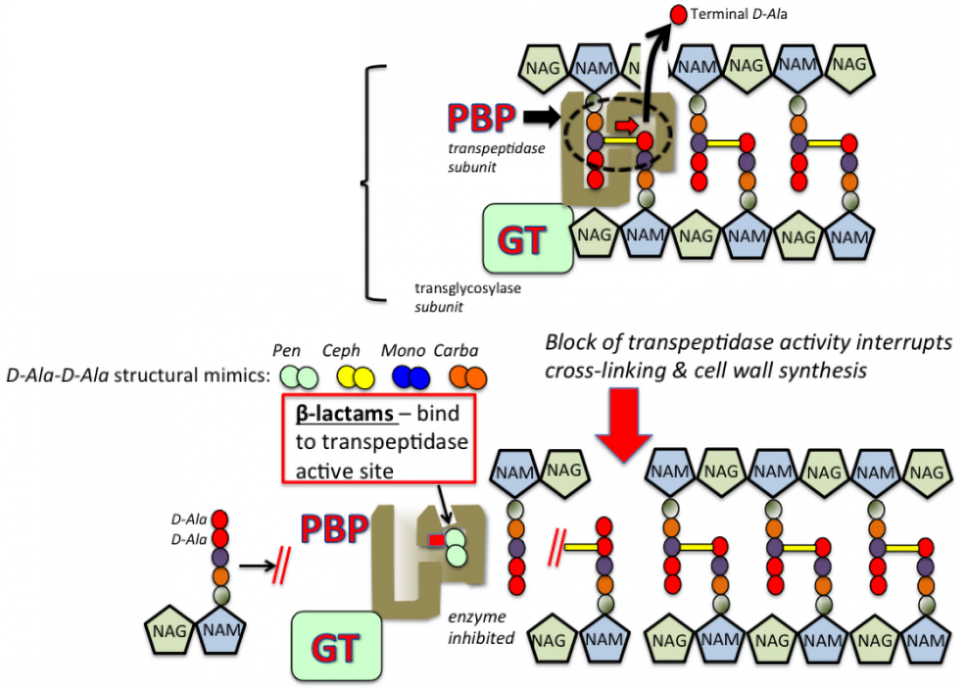

Figure 1.3: Mechanism of action of carbapenems.

1.2.1 Mechanism of carbapenem resistance in *Klebsiella pneumoniae*

Carbapenem resistance arises from three main mechanisms: (i) porin-mediated resistance to decrease uptake of carbapenems by deficiency of porin expression, or (ii) efflux pumps to reduce carbapenem inside of bacterial cell, or (iii) enzyme-mediated resistance with acquisition of carbapenemase genes that encode carbapenem-hydrolyzing enzymes [39, 40]. The reduced uptake through porin loss, or increased efflux of carbapenem are generally associated with an overexpression of β-lactamases that possess very weak affinity for carbapenems [27]. Three mechanisms of carbapenem resistance are shown in Figure 1.4.

K. pneumoniae has three main porin proteins in the outer membrane: OmpK35, -36, and -37, particularly OmpK35 and OmpK36 are responsible for the penetration of antibiotics to inside the cell [41]. *K. pneumoniae* regulates expression of porin proteins in the outer membrane to minimize the carbapenem entry into the periplasmic space where it binds to PBPs [42]. Combination of plasmid-encoded AmpC or another broad-spectrum β-lactamase expression together with decreased cell membrane permeability owing to

modifications or expression loss in OmpK35/36 and PhoE, phosphate transport porin, confer high-level carbapenem resistance in *K. pneumoniae* [41, 43]. An ertapenem-resistant ESBL producing K. pneumoniae carrying a novel OmpK36 porin variant has been reported in Italy and were found to be disseminated widely. Recent studies demonstrated that OmpK36 porin variant causes the ertapenem resistance and reduction in meropenem, and imipenem susceptibility [43].

acrAB and kexD from resistance/nodulation/cell division (RND) family; the kdeA efflux gene from multi drug and toxic compound extrusion (MATE) family; the kmrA gene from major facilitator superfamily (MFS) family; and kpnEF from small multidrug resistance (SMR) family are multidrug efflux systems functionally characterized in *K. pneumoniae* so far [44-46]. Each efflux pump family plays a role in antibiotic resistance but RND efflux pumps are a major mechanism of multidrug resistance in *K. pneumoniae* [47, 48]. The overexpression of acrAB efflux pump contributes to quinolone resistance, tigecycline resistance, and confer reduction in susceptibility to ertapenem and meropenem [44, 49, 50].

Carbapenem resistant non-cabapenemase producing strains are generally less resistant to other group antibiotics such as quinolone, aminoglycosides, polymyxins etc. The carbapenem resistance trait of these isolates cannot be transferred, unlike the strains possessing carbapenemase genes. Carbapenem resistant cabapenemase producing strains pose more clinical problems than non-carbapenemase producing strains (Nordmann [27]).

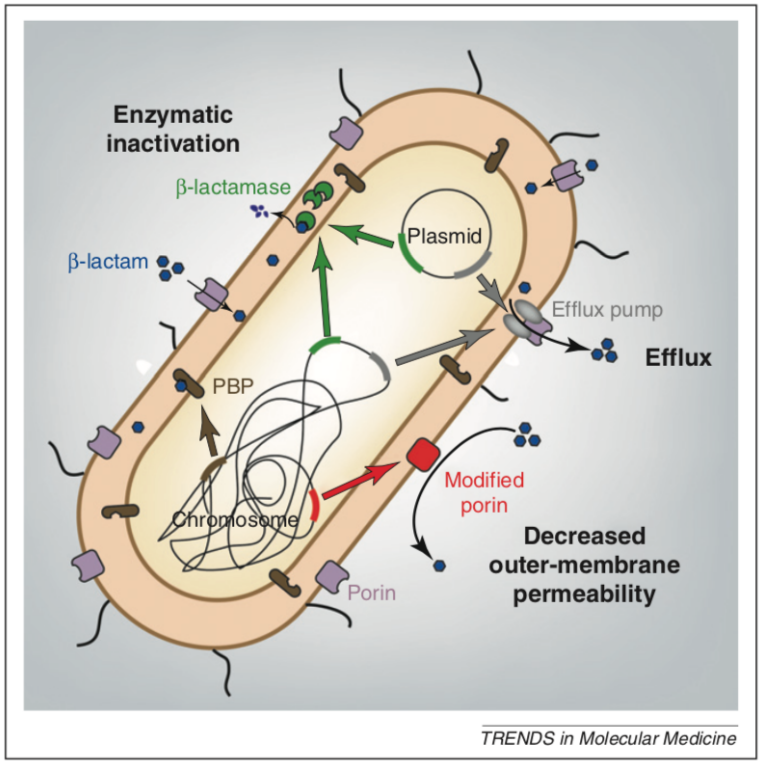

Figure 1.4: Mechanism of carbapenem resistance in *K. pneumoniae*. (i) porin-mediated resistance, (ii) efflux pumps, (iii) enzyme-mediated resistance

1.2.2 Cabapenemases

Cabapenemases that are versatile β-lactamases, hydrolyze β-lactam group antibiotics with few exceptions as well as hydrolyze carbapenem [22, 51]. Carbapenemase genes are carried on transposable genetic elements and plasmids which can be horizontally transferred rapidly between bacteria, thus they may spread all over the world [52]. SME-1 in London in 1982 and IMI-1 in the USA in 1984 were the first carbapenemases identified in *Enterobacteriaceae* [53, 54]. However, since then, widespread acquisition of carbapenemase genes have led dissemination of carbapenem-resistant *Enterobacteriaceae* worldwide [22].

Carbapenemases belong to three classes of β-lactamases based on the more commonly used Ambler classification system; class A, B and D. Class B that are called metallo-β-lactamases (MBLs) possess zinc residue at the active site to mediate bond hydrolysis. Classes A and D comprise serine β-lactamases (SBLs), the active site of which use serine

residue to facilitate ring opening [19, 22]. Some β-lactamase inhibitors (e.g., clavulanic acid, sulbactam, and tazobactam) inhibit SBL. However, MBLs can be inhibited only by metal ion chelators, such as dipicolinic acid, EDTA etc., not by β-lactamase inhibitors [28]. Chromosome-encoded cephalosporinases belonging to Ambler class C generally have slight extended activity toward carbapenems, thus clinical significance of Ambler class C remains debatable [22, 52, 55].

1.2.2.1 The Ambler class A carbapenemases in *Klebsiella pneumoniae*

NmcA/IMI, SME, and KPC carbapenemases that are three major types of class A carbapenemases hydrolyze all β-lactams except cephamycins [4, 27]. GES type b-lactamases that are the fourth member of class A was first considered as ESBL since GES-1 does not have carbapenemase activity. On the other hand, GES-1 variants appeared to have significant carbapenemase activity [4, 27].

K. pneumoniae carbapenemases (KPCs) are currently the most clinically troublesome enzymes among class A because of its location on self-conjugative plasmids [56]. The first KPC- producing strain (KPC-1) was isolated from *K. pneumoniae* in North Carolina in 1996 [57]. Soon thereafter, outbreaks of *K. pneumoniae* producing KPC-2 and KPC-3 were reported in New York [58, 59]. KPC-2 and KPC-3 continued to be disseminated in the East coast states of US and also, between *Enterobacteriaceae* as a consequence of horizontal gene transfer [60]. Within a few years, KPC-producing *K. pneumoniae* strains were reported worldwide in North and South Americas, the Middle East, Greece, Italy, and China where they are now considered endemic [4, 27, 61]. Also, sporadic spread of KPC-producing *K. pneumoniae* was reported in many European countries including Spain, France, Germany, the Netherlands, the UK, Ireland, Belgium, Sweden, and Finland, and in several countries in the Asia-Pacific region, including India, South Korea, and Australia [61]. Up to today, more than 20 different KPC variants have been identified. KPC-2 and KPC-3 remain the most common [55].

KPC-producing *K. pneumoniae* has disseminated in most countries because of expansion of a single dominant strain, ST258 [62, 63]. *K. pneumoniae* ST258 harboring KPC−2 is the prevalent clone reported in European countries and the USA, ST11, that is genetically related to ST258, is the predominant clone in KPC-producer *K. pneumoniae* in Asia [53,

62, 63]). Moreover, ST37, ST392, ST395 clones producing KPC-2 associated with nosocomial outbreak have been reported in China [53]. Although as compared to NDM-1 and OXA-48-like carbapenemases, the frequency of KPC-producer *K. pneumoniae* is not high in the Arabian Peninsula, two ST14 strains possessing KPC-2 have been first identified in the United Arab [64]. The coproduction of KPC and other carbapenemases in *K. pneumoniae* has been reported globally including KPC-2/NDM-1 in China; KPC-2/VIM-1 in Italy and Greece; and KPC-2/IMP-4 in China [65-67].

1.2.2.2 The Ambler class B carbapenemases (Metallo-β-Lactamases) in *Klebsiella pneumoniae*

Class B carbapenemases have hydrolytic activity for all β-lactam antibiotics with the exception of monobactams [68]. The first MBLs as chromosomal enzymes were isolated from environmental and opportunistic bacteria [69, 70]. The chromosome-borne metalloenzymes have not extensively contributed to an epidemiological burden of MBLs since they are not easily transferable [70]. However, association of integrons with transposable elements or plasmids facilitated acquisition o of MBL genes of bacteria and since the 1990s, considerable increase in MBL genes have been reported in *Enterobacteriaceae* [71]. The MBL families commonly identified in *Enterobacteriaceae* include IMP, VIM, and NDM groups [72].

The spread of NDM globally is one of the most problematic antibiotic resistances originated by MBLs. In 2008, the first NDM, NDM-1, was isolated from CRKP which was isolated from urine of a Swedish patient who was previously hospitalized in New Delhi [73]. *K. pneumoniae* harboring NDM is endemic in India, Pakistan, and Bangladesh but it sporadically has spread worldwide in the USA, European, Africa, Asia, and the Middle East countries [74]. While the Indian subcontinent remains the main reservoir, the Balkan states, the Middle East, and North African countries also may an additional reservoir based on the recent reports [75, 76]. So far, 24 distinct NDM variants have been identified [77]. NDM-1 was the most common carbapenemase type detected in India, Singapore, and the United Arab Emirates [78-80]. While ST11, ST14, ST15, ST147 or ST340 clones of NDM-producer *K. pneumoniae* has been most prevalent in many countries, NDM genes have been carried by various *K. pneumoniae* clones [77]. In India, England and Sweden, clinical *K. pneumoniae* isolates possessing NDM-1 have been

reported to mostly belong to the ST14, ST11, ST149, ST231 and ST147 clones [81]. NDM-producer *K. pneumoniae,* belonging to various sequence types (ST16, ST258, ST340, ST512, and ST972) which were imported from India through travelers has been reported in Canada [82]. Also, ST20 and ST17 producing NDM-1 associated with an outbreak in neonatal ICU was identified in China [83]. The coproduction of NDMs and other carbapenemases in *K. pneumoniae* has also been reported worldwide including NDM1/OXA-48 in Turkey, United Arab Emirates, Australia, Morocco, and Switzerland; NDM-1/OXA-232 in India and USA [74].

1.2.2.3 The Ambler class D carbapenemases (Oxacillinases) in *Klebsiella pneumoniae*

Class D β-lactamases also called oxacillinases (OXAs) commonly hydrolyze isoxazolyl penicillins [84]. Even OXAs consist of more than 400 enzymes, a few variants possess carbapenemase activity, but they do not hydrolyze expanded-spectrum cephalosporins [4, 27]. OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-58-like, OXA-143-like, and OXA-235-like families are main OXA enzymes having carbapenem-hydrolyzing activity [71].

Although most of these groups have been detected in *Acinetobacter* spp., the first OXA-48 enzyme was isolated from imipenem resistant *K. pneumoniae* in Turkey in 2003 [85] and so far, 10 variants of OXA−48 gene have been identified [4, 27, 68]. Since 2003, Turkey has been reported as possessing the highest epidemiologic level of *K. pneumoniae* OXA-48 producing strains and followed by India, Middle East, and North African countries [4, 27]. Also, sporadic spread has been reported in several European countries, including France, Germany, Netherlands, Italy, Belgium, UK, Ireland, Slovenia, Switzerland, and Spain, in the Middle East, and in Japan [74, 85]. The frequency of OXA-48 carbapenemases among carbapenemase-producer *K. pneumoniae* has been found significantly high in Spain, France, Romania, African countries, and Arabian Peninsula [74]. OXA-181 derived from OXA-48 was first identified in India and then has spread to many countries including UK, Romania, Canada, Oman, Singapore, Sri Lanka, South Korea, Australia, Japan, and New Zealand [74]. Moreover, OXA-232, another derivative of OXA-48, harboring *K. pneumoniae* has been reported in various countries such as the USA, Singapore, India, and South Korea [74]. OXA-48 together with the derivatives remain to spread worldwide. While ST11 clones of OXA-48-like producer *K.*

pneumoniae has been most prevalent in many countries, Spain, Taiwan, Libya, Turkey, Argentina, and Greece, OXA-48-like genes have been identified in various *K. pneumoniae* clones [74]. Also, ST14, ST15, ST101, ST147, and ST405 possessing OXA-48-like frequently have been reported in many countries. Molecular epidemiology studies in European and north-African countries showed that ST101 clone of *K. pneumoniae* isolates was the most frequently detected clonal type, followed by ST395 and ST15 [86].

The coproduction OXA-48-like and other carbapenemases in *K. pneumoniae* was also often reported worldwide including OXA-48/NDM-1 in Turkey, Switzerland, United Arab Emirates, Australia, Morocco; OXA-181/NDM-1 in Singapore; and OXA-232/NDM-1 in the USA and India [74].

1.3 Bloodstream infections originated by *Klebsiella pneumoniae*

The dissemination of carbapenem-hydrolyzing enzymes and extensive use of carbapenems have led to spread of carbapenemase producing carbapenem resistant *K. pneumoniae* (CRKP) worldwide which has become a crucial public health problem [87]. Bloodstream infection is the most important infection caused by CRKP with serious conditions and a poor prognosis [88]. The mortality rate of CRKP BSI was determined as 71.9% which was much higher with 21.9% than CRKP infection in other sites in a case-control study [89].

Resistance to multiple antibiotics caused by possessing carbapenemase reduces the probability of patient survival by limiting effective and safe treatment options. The mortality rate of patients who have bloodstream infections originated by CRKP is 40% to 70%, while this rate is 20% to 30% for patients infected by carbapenem susceptible strains [9]. Colistin has been utilized as a last resort antibiotic for treatment of infections originated by CRKP [90]. Global dissemination of carbapenemase producer *K. pneumoniae* has led to increased use of colistin and thus emergence of colistin resistance in CRKP [90]. The increasing prevalence of colistin-resistance in CRKP has increased mortality in *K. pneumoniae* bloodstream infections by further reducing therapeutic options [91]. The improvement of survival of patients with CRKP BSIs with combinational therapy including carbapenem has raised some controversy. The recent study focused on treatment regimens for CRKP BSI showed that use of combinational

therapy including meropenem, or an extended-spectrum cephalosporin does not significantly affect survival of patients compared to monotherapy [5, 92, 93]. In addition, a study of ceftazidime-avibactam treatment against CRKP BSIs showed that the rates of clinical success were significantly higher among patients receiving ceftazidime-avibactam (85%) than among patients receiving combinational therapy including carbapenem/aminoglycoside (48%) and carbapenem/colistin (40%) [94].

Considering the increase in CRKP bloodstream infections and associated increase in mortality, in this study, we aimed to describe molecular epidemiology of CRKP bacteremia in Turkey and to evaluate the effect of carbapenemase producing high risk clones on mortality.

Chapter 2:
METHODS

2.1 Study population

In this multicenter study, 254 adult patients with CRKP BSIs hospitalized in 13 tertiary care centers (from İstanbul Koç University Hospital, Cerrahpaşa University Hospital, İstanbul Training and Research Hospital, Koşuyolu State Hospital, Okmeydanı Training and Research Hospital, Marmara University Pendik Training and Research Hospital, Florence Nightingale Hospital, Sultan Abdülhamid Han Training and Research Hospital; from Ankara Başkent University Hospital, Ankara Training and Research Hospital, Hacettepe University Hospital, Ankara University Hospital; from Bursa Uludağ University Hospital) in Turkey between June 2018 and June 2019 were included. Carbapenem resistance was defined as if *K. pneumoniae* is non-susceptible to at least one carbapenem based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2018 breakpoints (MIC 2 µg/mL for meropenem or imipenem, and 0.5 µg/mL for ertapenem) [95]. 30-day mortality for the patients was recorded by following the patients up for 30 days after the first CRKP positive blood culture was collected. The study was approved by the Koç University Institutional Review Board with the approval number:2018.151.IRB1.018. A web-based data entry was utilized for data recording.

2.2 Bacterial identification and antibiotic susceptibility testing

Bacterial identification and first carbapenem antibiotic susceptibility testing were performed using automated systems (i.e., BD Phoenix or VITEK 2) at local laboratories. After identification of the isolates as carbapenem resistant *K. pneumoniae*, isolates were transferred to the reference microbiology laboratory for further antibiotic susceptibility testing and molecular studies.

2.2.1 Meropenem susceptibility testing with Etest

The minimum inhibitory concentration (MIC) of meropenem was re-tested by the Etest (BioMérieux, France) method. The isolates were grown overnight on Tryptic Soy Agar (TSA) (Becton, Dickinson and Company, USA) at 37 ^{o}C. From each isolate, a McFarland 0.5 bacterial suspension in cation-adjusted Mueller Hinton II Broth (MHB) (Becton,

Dickinson and Company, USA) was prepared. A sterile cotton swab moistened with the 0.5 McFarland bacterial suspension was inoculated to cation-adjusted Mueller-Hinton II agar plates and Etest strips were placed to agar plates. After overnight incubation at 37 ºC, the MIC was read where the elliptical zone of inhibition intersected the Etest strip [96]. Meropenem resistance was determined based on EUCAST Guideline [95].

2.2.2 Colistin susceptibility testing with broth microdilution method

The minimum inhibitory concentration of colistin was determined by the broth microdilution method (BMD). The isolates were grown overnight on TSA at 37 ºC. 0.5 McFarland bacterial suspension in 2 ml of cation-adjusted MHB was prepared from each isolate. 100 µl of cation- adjusted MHB was added into each well of a polystyrene flat bottom 96-well plate. 100 µl of 128 µg/mL colistin prepared in cation-adjusted MHB was transferred to first column of 96-well plate and 2-fold serial dilution was performed by pipetting up and down except the last column, thus colistin concentrations from 64 µg/mL to 0 µg/mL were obtained. 10 µl of bacterial suspension was inoculated to each well and each isolate was duplicated. After overnight incubation at 37 ºC, the MIC_{90} was read where it inhibits 90% of bacterial growth. MICs were categorized according to EUCAST [95].

2.2.3 Ceftazidime-avibactam, meropenem, and colistin susceptibility testing using Sensititre EURGNCOL plates

The minimum inhibitory concentrations of ceftazidime-avibactam, meropenem and colistin were determined using Sensititre EURGNCOL plates (Thermo Fisher, U.S.) according to the manufacturer's recommendations. The isolates were grown overnight on TSA at 37 ºC. 0.5 McFarland bacterial suspension in 2 ml of distilled water was prepared from each isolate. 30 µl of bacterial suspension was added to cation-adjusted MHB. 50 µl of mixture was inoculated to each well of Sensititre plate and each isolate was studied in duplicate. After overnight incubation at 37 ºC, the MIC_{90} was read. Ceftazidime-avibactam, meropenem, and colistin resistance were determined based on EUCAST Guideline [95].

In all antibiotic susceptibility tests, *E.coli* ATCC 25922 standard strain was used as reference control strain. The antimicrobial susceptibility breakpoints of meropenem,

colistin, and ceftazidime-avibactam for *K. pneumoniae* were summarized in Table 2.1 [95].

Table 2.1: The antimicrobial susceptibility breakpoints (EUCAST 2018 Guideline)

Antimicrobial	Susceptible (S≤)	Resistant (R>)
meropenem	2	8
colistin	2	2
ceftazidime/avibactam	8	8

2.3 Molecular Analysis

Total DNA isolation from isolates was performed in order to use for further molecular analysis. After overnight growth on TSA at 37°C, DNA was isolated using a commercial DNA extraction kit according to the manufacturer's instructions (QIAGEN DNeasy UltraClean Microbial Kit, USA). Briefly, the bacterial cell wall was disrupted with powerbead and cellular components were lysed with lysis buffer. After the washing step, bacterial DNA was eluted by 50 µl of the elution buffer. DNA isolated was stored at -20°C to be further used in determination of the clonal relationships of the clinical isolates by pulsed-field gel electrophoresis (PFGE), in identification of sequence types of isolates by MLST, and in detection of carbapenamase genes by multiplex PCR.

2.3.1 Pulsed-Field Gel Electrophoresis (PFGE)

The pulsed-field gel electrophoresis was performed in Ankara University Medical Microbiology Laboratory to evaluate clonal relatedness of the isolates. The overnight-grown bacterial cultures were adjusted to OD_{600} 0.9 in EET Buffer. For solidification, low-melting-temperature agarose (2%) was added to bacterial suspensions in equal volumes. Then solid agarose was incubated for 4 h at 37°C in an EET buffer including 1 mg/ml lysozyme and 50 µg/ml lysostaphin. The plugs in the EET buffer including 1% sodium dodecyl sulfate (SDS) and 20 mg/ml proteinase K buffer were incubated

overnight at 50°C. After incubation, the plugs were digested overnight with XbaI restriction endonuclease (NEB, USA) [83]. DNA separation was performed using pulsed-field electrophoresis. Computer analysis of the DNA bands separated was performed using the BioNumerics fingerprinting software (Applied Maths, Belgium; http://www.applied-maths.com/bionumerics).

2.3.2 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was performed for a representative strain of each PFGE type by amplification of seven housekeeping genes: *phoE, gapA, rpoB, tonB, inf, mdh,* and *pgi*. Amplification reactions were carried out in Applied Biosystems Veriti 96 Well Thermal Cycler (Applied Biosystems, USA) using 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 $MgCl_2$. Amplification-PCR mix containing 12.5 µl of DreamTaq Green PCR master mix, 5.5 µl of Betaine, 5.5 µl of nuclease free water, and 0.5 µl of 25 pmol forward and reverse primers (Table 2.2.), and 0.5 µl of DNA isolated was prepared for each housekeeping gene. Following reaction conditions were used; initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 20 sec, annealing at 50°C for *rpoB*; 55°C for *mdh*, *pgi*, *infB*; 59°C for *tonB*; and 60°C for *gapA* and *phoE* for 30 sec, extension at 72°C for 30 sec, final extension at 72°C for 5 min and ending at 4°C. After amplification, amplicons were run for 30 min at 90 V on 1.5% agarose gel.

Amplified products of *phoE, gapA, rpoB, tonB, inf, mdh,* and *pgi* were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Purified products were amplified using BigDye Terminator v3.1 Cycle (Applied Biosystems, U.S.) which amplifies products based on dideoxy-chain termination method. Amplification-PCR mix containing 2 µl of BigDye Terminator v3.1 Cycle, 1 µl of BigDye Buffer, 12.8 µl of nuclease free water, 3.2 µl of 1 pmol forward and reverse primers (Table 2.2.), and 1 µl of purified product was prepared for each gene. 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 amplified products were purified using ZR-96 DNA Sequencing Clean-up Kit (Zymo Research, U.S.) and were sequenced by Applied Biosystems ABI 3500 Genetic Analyzer. ABI files of gene sequences were analyzed and

allelic profiles of isolates for each gene were determined in Applied Maths Bionumerics version 7.6 Software (Biomerieux, France). The sequence types (STs) of isolates from a combination of allelic profiles of each gene were identified using the Institute of Pasteur website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

2.3.3 Carbapenamase Typing PCR

Carbapenamase genes ($bla_{OXA\text{-}48\text{-}like}$, $bla_{KPC\text{-}2}$, $bla_{NDM\text{-}1}$) were detected by multiplex PCR. Amplification-PCR mix containing 12.5 µl of DreamTaq Green PCR master mix, 3 µl of Betaine, 3 µl of nuclease free water, and 1 µl of 25 pmol forward and reverse primers of each gene (Table 2.2.), and 0.5 µl of DNA isolated was prepared. Following reaction conditions were used; initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 20 sec, annealing at 62°C for 30 sec, extension at 72°C for 30 sec, final extension at 72°C for 5 min, and ending at 4°C. Amplicons were separated by running for 30 min at 90 V on 1.5% agarose gel and carbapenemase genes were determined based on their amplicon size using 100 kb DNA ladder.

2.4 Statistical Analysis

Survivors and deceased patients were compared to identify the factors associated with 30-day mortality. Continuous variables were compared using t-test or Mann-Whitney U test. Categorical variables were compared with Pearson's $\chi 2$ test. Two-tailed tests were used to determine statistical significance and a p value of ≤ 0.05 was considered significant. Univariate cox regression analyses were performed to evaluate the effect of predefined covariates on 30-day mortality. All analyses were performed using STATA software version 16.0.

Table 2.2: The primers used in molecular analysis

Oligonucleotide	Sequence	Used for	Amplicon size (bp)
rpoB-F	GGCGAAATGGCWGAGAACCA	PCR/Sequence	1089
rpoB-R	GAGTCTTCGAAGTTGTAACC		
gapA-F	TGAAATATGACTCCACTCACGG	PCR/Sequence	700
gapA-R	CTTCAGAAGCGGCTTTGATGGCTT		
mdh-F	CCCAACTCGCTTCAGGTTCAG	PCR/Sequence	757
mdh-R	CCGTTTTTCCCCAGCAGCAG		
pgi-F	GAGAAAAACCTGCCTGTACTGCTGGC	PCR/Sequence	730
pgi-R	CGCGCCACGCTTTATAGCGGTTAAT		
phoE-F	ACCTACCGCAACACCGACTTCTTCGG	PCR/Sequence	604
phoE-R	TGATCAGAACTGGTAGGTGAT		
infB-R	CTCGCTGCTGGACTATATTCG	PCR/Sequence	318
infB-R	CGCTTTCAGCTCAAGAACTTC		
tonB-F	CTTTATACCTCGGTACATCAGGTT	PCR/Sequence	414
tonB-R	ATTCGCCGGCTGRGCRGAGAG		
OXA-48-like-F	GCGTGGTTAAGGATGAACAC	PCR	438
OXA-48-like-R	CATCAAGTTCAACCCAACCG		
NDM-1-F	GGTTTGGCGATCTGGTTTTC	PCR	621
NDM-1-R	CGGAATGGCTCATCACGATC		
KPC-2-F	CGTCTAGTTCTGCTGTCTTG	PCR	798
KPC-2-R	CTTGTCATCCTTGTTAGGCG		

Chapter 3:
RESULTS

3.1 Bacterial identification

Two hundred thirty-six (93%) of 254 isolates collected from different healthcare centers were speciated as *K. pneumoniae* at the reference microbiology laboratory using MALDI-TOF MS. The remaining 18 isolates identified as *K. pneumoniae* at the local laboratories were identified as *K. variicola* (n=15), *K. quasipneumoniae* (n=2), and *K. oxytoca* (n=1). The entire *Klebsiella sp.* were termed as "CRKP".

3.2 Carbapenem susceptibility and carbapenemase production

As shown in Table 3.1., the discordance for meropenem susceptibility between the automated systems and broth microdilution/Etest was detected. Twenty-six (10%) of 254 isolates were found to be meropenem susceptible by automated systems in local laboratories. However, 51 (20%) isolates were detected as meropenem susceptible by both broth microdilution and E test methods at the reference microbiology laboratory. In total 51 (20%) of 254 CRKP isolates were determined as carbapenem susceptible because they were also detected as susceptible to other members of the carbapenem with an automated system, and they were tested negative for all known carbapenemases. Although one isolate was an OXA-48-like producer, this isolate was determined as carbapenem susceptible. Meropenem MICs of carbapenem susceptible *Klebsiella pneumoniae* (CSKP) isolates determined by broth microdilution and Etest methods were summarized in Table 3.2.

Table 3.1: Meropenem susceptibility of 254 CRKP isolates

	Meropenem susceptibility (n=254)	
	Resistant (n, %)	Susceptible (n, %)
Automated system	228 (90%)	26 (10%)
Etest	203 (80%)	51 (20%)
Broth microdilution	203 (80%)	51 (20%)

One hundred eighty-seven (92%) of 203 CRKP isolates were carbapenemase producing carbapenem resistant strains and the remaining 16 isolates were non-carbapenemase producing carbapenem resistant strains. OXA-48-like carbapenemases comprised the largest carbapenemase group with 171 isolates (91%). Thirty isolates were OXA-48-like carbapenemase and NDM-1 co-producer. Ten isolates produced a single NDM-1 and four isolates harbored a single KPC-2. NDM-1 was accompanied by KPC-2 in two isolates. The carbapenemase characteristics of CRKP isolates were demonstrated in Table 3.3.

3.3 Clonal relatedness of the clinical isolates

The dendrogram of the isolates was shown in Figure 3.1. The isolates which have more than 95% similarity were accepted as the members of the same clone, based on the similarity index. As shown in the dendrogram, isolates belonged to 80 different clones. While each of 55 clones represented one strain, the remaining 25 clones consisted of more than one strain. Clone A and B constituted the major clonal types.

Table 3.2: Meropenem MICs of CSKP isolates determined by broth microdilution and Etest methods

Isolate no	Meropenem MIC with Etest	Meropenem MIC with Broth microdilution	Meropenem MIC with automated system	Isolate no	Meropenem MIC with Etest	Meropenem MIC with Broth microdilution	Meropenem MIC with automated system
1	0.012	1	4	**27**	0.016	0.5	8
2	0.19	<0.25	8	**28**	0.012	0.5	8
3	0.047	1	4	**29**	0.008	0.5	8
4	0.047	<0.25	8	**30**	0.012	0.5	32
5	0.125	<0.25	32	**31**	0.008	0.5	4
6	0.047	1	32	**32**	0.006	0.5	4
7	0.125	<0.25	2	**33**	0.006	0.5	4
8	0.125	<0.25	8	**34**	0.032	0.5	4
9	0.19	<0.25	4	**35**	0.016	0.5	4
10	0.38	<0.25	4	**36**	0.016	0.5	4
11	0.094	<0.25	8	**37**	0.012	0.5	4
12	0.023	2	>16	**38**	0.008	0.5	16
13	0.094	<0.25	>8	**39**	0.047	0.5	4
14	0.25	<0.25	>16	**40**	0.5	0.5	4
15	0.25	<0.25	>8	**41**	0.047	0.5	4
16	0.12	0.5	32	**42**	0.064	0.5	4
17	0.12	0.5	32	**43**	0.25	0.5	4
18	0.12	0.5	32	**44**	0.5	0.5	2
19	0.032	0.5	8	**45**	0.064	1	2
20	0.016	0.5	32	**46**	0.094	0.5	4
21	0.032	1	16	**47**	0.38	1	2
22	0.012	1	4	**48**	0.5	0.25	4
23	0.023	0.5	32	**49**	0.19	0.5	32
24	0.006	0.5	32	**50**	0.047	0.5	>32
25	0.012	0.5	2	**51**	0.19	<0.25	4
26	0.012	0.5	2				

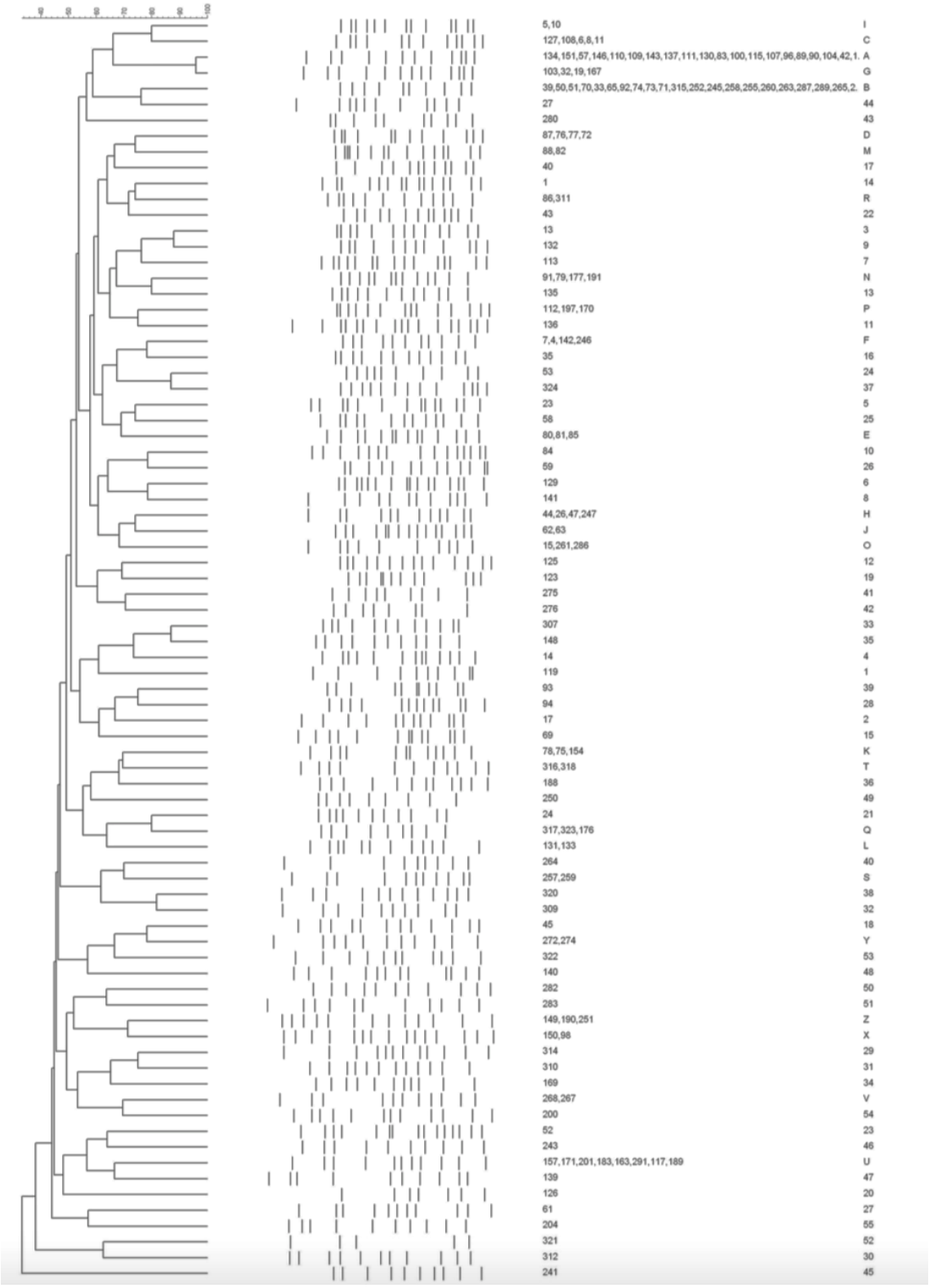

Figure 3.1: Dendrogram of 254 isolates. Similarity index scale is located above the dendrogram. The isolates are enumerated, and PCR gel images of the isolates are shown next to the dendrogram.

3.4 Multi-locus sequence types

MLST was performed for representative strains of each clone type. As shown in Figure 3.2., among 203 CRKP isolates, ST2096 constituted the largest clonal type with 62 isolates (30%) followed by ST101 with 36 isolates (18%) and ST14 with 27 isolates (13%). Remaining isolates were dispersed among various clones including ST16 (10%), ST307 (3%), ST981 (3%), ST11 (2%), ST15 (2%), ST395 (2%), ST147 (1%), ST187 (1%), and ST111 (1%). Fifty-one CSKP strains mainly belonged to ST187 with 18 isolates (35%), ST250 with 8 isolates (16%) and ST111 with 7 isolates (14%), and ST551 with 6 isolates (12%). Remaining CSKP isolates belonged to ST16 (6%), ST2096 (4%), ST307 (2%) and ST147 (2%).

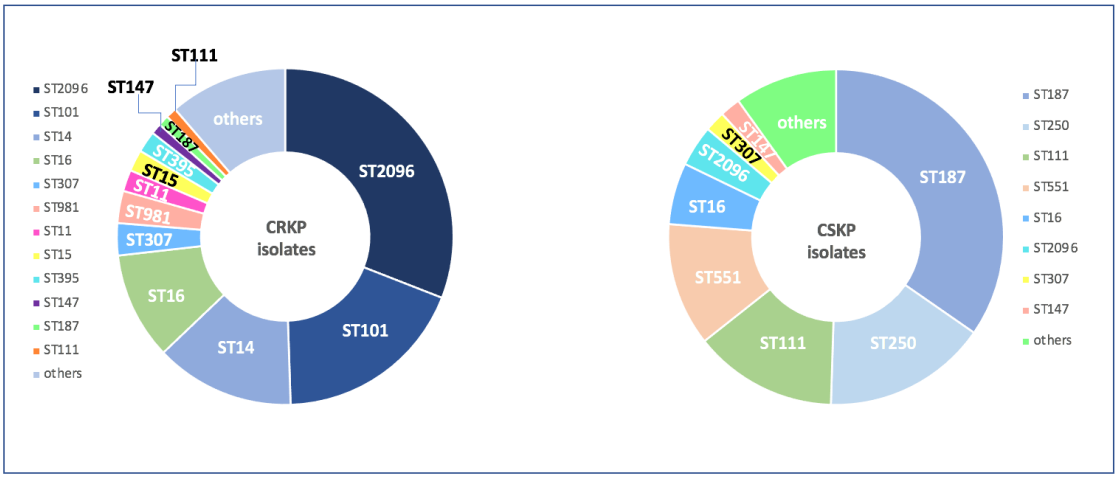

Figure 3.2: Sequence types of 203 CRKP and 51 CSKP isolates.

Table 3.3. demonstrates the carbapenemase characteristics and sequence types of CRKP isolates. OXA-48-like carbapenemases were mainly carried on ST2096 in 61 isolates (61/171, 35%), and on ST101 in 35 isolates (35/171, 20%). The remaining OXA-48-like carbapenemase producers were dispersed among various clonal types including ST14 (15%), ST16 (8%), ST307 (2%), ST981 (4%), ST11 (2%), ST147 (1%), and ST187 (1%). Majority (19 of 30) of OXA-48-like/NDM-1 co-producers (63%) were carried on ST14 and remaining co-producers belonged to ST2096, ST16, ST15 and ST101. NDM-1/KPC-2 co-producers were carried on ST15.

Table 3.3: Carbapenemase ($bla_{OXA-48-like}$, bla_{KPC-2}, bla_{NDM}) characteristics and sequence types of CRKP isolates

	CRKP isolates						
ST	**Total**	**OXA-48-like**	**OXA-48-like/NDM-1**	**NDM-1**	**KPC-2**	**KPC-2/NDM-1**	**No carbapenemase**
ST2096	62	58	3	-	-	-	1
ST101	36	34	1	-	-	-	1
ST14	27	7	19	1	-	-	-
ST16	21	12	2	-	-	-	7
ST307	7	4	-	-	3	-	-
ST981	6	6	-	-	-	-	-
ST11	5	3	-	2	-	-	-
ST15	5	1	1	1	-	2	-
ST395	5	1	-	4	-	-	-
ST147	3	2	-	-	-	-	1
ST187	2	2	-	-	-	-	-
ST111	1	-	-	-	-	-	1
Other	23	11	4	2	1	-	5
Total	203	141	30	10	4	2	16

3.5 Colistin and ceftazidime/avibactam susceptibilities

As shown in Table 3.4., overall colistin susceptibility was 38% (97 of 254 isolates). The ST101 and ST2096 clonal types had high colistin resistance rates with 83% and 81%, respectively. Majority of the CRKP isolates (92%) were determined as colistin susceptible as well as carbapenem determined by BMD method. Ceftazidime-avibactam was the most active antibiotic with 84% susceptibility (213 of 254 isolates). However, isolates belonging to ST14 clonal type had the lowest susceptibility with 22% (6 of 27 isolates) against ceftazidime-avibactam.

Table 3.4: Colistin and ceftazidime/avibactam susceptibilities of isolates (data in n (%); BMD, broth microdilution). *E.coli* ATCC 25922 standard strain was used as reference control strain.

Susceptibility (susceptible/total tested)	**Total**	**CRKP isolates**				**CSKP isolates**
		ST2096	**ST101**	**ST14**	**ST16**	
Meropenem BMD	65/254 (26%)	3/62 (5%)	1/36 (3%)	0/27 (0)	9/21 (42%)	0/51(0)
Colistin, BMD	97/254 (38%)	12/62 (19%)	6/36 (17%)	10/27 (37%)	8/21 (38%)	47/51 (92%)
Ceftazidime-avibactam, BMD	213/254 (84%)	61/62 (98%)	35/36 (97%)	6/27 (22%)	18/21 (86%)	51/51 (100%)

3.6. Thirty-day mortality rates

The 30-day all-cause mortality was calculated with Pearson X2 test (P=0.038) as 40% (102 of 254) for all patients, as 43% (88 of 203) for infected with the CRKP population and as 27% (14 of 51) for infected with the CSKP population. Mortality rates in infections with the ST2096, ST14 and ST101 clonal groups were 50% (31 of 62), 53% (19 of 36) and 41 % (11 of 27), respectively. The factors associated with 30-day mortality were demonstrated in Table 3.5. The non-OXA-48-like carbapenemase type (P=0.04), and ST2096 (P=0.02) and ST14 (P=0.002) clonal types were found to be associated with 30-day mortality with the univariate cox regression analysis. Carbapenem resistance in

CRKP and CSKP groups was not found to be associated with mortality in univariate (HR 1.62, 95% CI 0.92-2.85, P=0.10) analyses.

Table 3.5: The factors associated with 30-day mortality (HR, hazard ratio; CI, confidence interval).

	Univariate analysis		
	HR	**95% CI**	**P**
Carbapenem resistance	1.62	0.92-2.85	0.1
Carbapenemase type			
OXA-48-like	1.49	0.82-2.71	0.19
OXA-48/MBL (n=30), MBL (n=10), KPC (n=4), KPC/MBL (n=2)	1.89	1.03-3.48	0.04
MLST type			
ST2096	1.77	1.09-2.88	0.02
ST101	1.24	0.64-2.39	0.64
ST14	2.59	1.43-4.68	0.002

Chapter 4:
DISCUSSION

Carbapenem resistant *Klebsiella pneumoniae* (CRKP) has become a clinically important bacteria, causing worldwide nosocomial infections with high rates of antibiotic resistance and mortality [13, 22, 23]. Carbapenem resistance in *K.pneumoniae* commonly spread worldwide through OXA-48-like, NDM and KPC carbapenemases [51]. The spread of CRKP has been associated with some specific clonal types with higher transmissibility rates and greater acquisition of antibiotic resistance genes [74]. Bacteremia which is the most important infection originated by CRKP is associated with high mortality rates, thus rapid spread of high-risk clones of carbapenemase producing CRKP that causes bloodstream infections (BSI) has been concerning [9, 88]. This study focuses on molecular epidemiology of CRKP bacteremia in Turkey. Up to today, several studies have been published on CRKP bacteremia mainly focused on the clinical outcome of patients or predictors of mortality, or appropriate antibiotic treatment options; however, these studies usually lack of detailed molecular characteristics of CRKP isolates [89, 92, 97, 98]. The inclusion of the CRKP isolates accompanied with the molecular data is the strongest part of this study.

The sporadic spread of NDM and endemic spread of OXA-48-like carrying *K. pneumoniae* has been reported in Turkey [99, 100]. The studies showed that OXA-48-like is the dominant carbapenemase type with 75%-96% frequency in Turkey [86, 101-103]. In this study, it was found that OXA-48-like comprised the largest carbapenemase group (91%, 171/187) as consistent with literature. In 2012, first NDM-1 producer *K. pneumoniae* was reported in Turkey, after this time, NDM producers have been increasingly reported [104]. While the rate of NDM producer *K. pneumoniae* was 4% in 2013, it has increased to 20% today [86, 103, 105, 106]. In this study, the rate of NDM-1 was also found as 21%.

Emerging high-risk CRKP clones causing BSI has been increasingly reported. The studies showed that CRKP isolates which cause BSI mainly belonged to ST11, ST258, ST374 ST512 worldwide [92, 107-109]. Loconsole et al. recently reported high risk ST101 and ST307 CRKP clonal types in Southern Italy, and Andrey et al. reported ST16 in Brazil [108, 110]. This study demonstrated the emergence and multi-hospital spread of an OXA-48-like carbepenemase carrying clonal lineage ST2096 (61/254, 24%). Three of 61

isolates (0.05%) also possessed NDM-1. NDM-1 producer *K. pneumoniae* belonging to ST2096 clone has not been reported previously. The OXA-48-like carbapenemase positive ST2096 type *K. pneumoniae* was first identified in India [111, 112]. In Saudi Arabia, ST2096 *K. pneumoniae* outbreak has been reported recently [113]. We suggested that OXA-48-like carrying ST2096 was probably imported to Turkey through international travel.

ST101 associated with high colistin resistance rate (83%) was the second most common clonal type in our population (36/254, 14%). In the previous studies in Turkey, ST101 was found to be a predominant clone with 59%-95% frequency in colistin resistant *K. pneumoniae* [86, 101]. In this study, OXA-48-like carbapenemase was detected in 35 of 36 (97%) isolates and one of these isolates was NDM-1 and OXA-48-like producer. In Turkey, NDM-1 producer ST101 *K. pneumoniae* has not been reported before.

ST14 was the third common clonal type with 13% frequency in our collection. The studies showed that ST14 is a single locus variant of ST2096 and is the most common clonal type in Sweden, the United Kingdom and India [81]. However, ST14 has not been frequently isolated up to today in Turkey. ST14 has been described as a host for the NDM-1 [81]. In this study, NDM-1 was detected in seventy percent of isolates belonging to ST14 (20/27) and nineteen of these isolates were OXA-48-like/NDM-1 co-producers. This is the first report for OXA-48-like and NDM-1 co-producer belonging to ST14 in Turkey. OXA-48-like/NDM-1 co-producer ST14 strains were first reported in a recent study in the United Arab Emirates showing half of these isolates belonged to ST14 clone [114].

Turkey is among the countries where have seen high colistin resistance rates in CRKP. After the first isolation of colistin-resistant *K. pneumoniae* in 2012, colistin resistance has increased over the years [115]. The colistin resistance rate in *K. pneumoniae* associated with bloodstream infections increased from 6% in 2013 to 16% in 2015 [116, 117]. In this study, overall colistin resistance was found as 62%. The ST101, ST2096 and ST14 clonal types had high colistin resistance rates with 83%, 81%, and 63% respectively. The high colistin resistance in ST101 type *K. pneumoniae* also was shown in previous study of our team [86]. Emerging ST2096 and ST14 clones have been associated with high colistin resistance in India and United Arab Emirates [112, 114]. The high colistin resistance in ST101, ST14 and ST2096 clones may have led them to become

predominated clonal types in Turkish hospitals by evolving and expanding under colistin pressure.

Ceftazidime-avibactam was the most active antibiotic against CRKP with overall 84% susceptibility (213/254) and 98% (208/211) in non-NDM-1 producers in this study. Since 74% of ST14 clone produced NDM-1, ceftazidime-avibactam resistance rate was 78% in ST14. Similar results were reported in a recent research, where 3.7% of CRKP isolates were resistant to ceftazidime-avibactam and among these isolates 53.1% were MBL-producer *K. pneumoniae* [118]. These findings confirm that MBL producers could not be treated with combination of beta-lactam and beta-lactamase inhibitor currently available. Moreover, Zhang et al. demonstrated that ceftazidime-avibactam monotherapy was not effective for infections originated by NDM-1 producer *K. pneumoniae* and combination with aztreonam is necessary for the treatment [118].

Resistance to carbapenem has limited the effective and safe treatment options, thus causing a reduction in probability of patient survival. As consistent with the literature, 30-day all-cause mortality was 40% (102 of 254) for all patients, 43% (88 of 203) for infected with CRKP, and 27% (14 of 51) for infected with CSKP (P=0.038) in this study. Mortality rates in ST101, ST2096, and ST14 clonal groups were 41% (11 of 27), 50% (31 of 62), and 53% (19 of 36) respectively. ST2096 and ST14 clones were found to be associated with 30-day mortality with univariate analysis, P= 0.02, and P=0.002, respectively. The colistin-resistance in these clones may have led to increased mortality by further reducing therapeutic options. The recent study showed that the mortality of patients infected with colistin resistant *K. pneumoniae* population which mainly consisting of ST101 was 61%, whereas mortality rate was 23% in patients infected with colistin susceptible *K. pneumoniae* [86].

Another finding of our study was the discordance for meropenem susceptibility between the automated systems and broth microdilution/Etest. The previous studies have shown that automated systems fail to detect carbapenem resistance and so miss some CRKP isolates [119-121]. However, in this study, 51 of 254 isolates (20%) that were reported as carbapenem resistant at local laboratories using mainly automated systems were determined as meropenem susceptible at the reference microbiology laboratory using broth microdilution and Etest methods (Table 3.1). Haffler et al. demonstrated that while

the susceptibility in carbapenemase producer *Enterobacteriaceae* was overestimated by an automated system, carbapenem resistance overcall in non-carbapenemase producing isolates were detected [120]. The CSKP strains in this study did not possess any of the known carbapenemase genes, this may be the reason why carbapenem resistance overcall by automated systems were detected in our collection.

The limitation of this study is screening the isolates only for OXA-48-like, NDM-1 and KPC-2 carbapenemases which are the most common carbapenemases responsible for the dissemination of carbapenem resistance global. The next generation sequencing of the isolates in future studies will provide detailed information on all known carbapenemases with their subgroups, mutations responsible for carbapenem resistance, and virulence factors associated with mortality.

In conclusion, this study demonstrated that CRKP belonging to high-risk clones were associated with high colistin resistance rate and high mortality rate. Emerging ST2096 possessing OXA-48-like is now the predominating clonal type in bloodstream infections in Turkish hospitals. Epidemiology of carbapenemases is shifting towards MBL and non-MBL co-producers, carried on high-risk clones including ST14, ST2096 and ST101, which constitute a major concern. High colistin resistance and mortality rates in clones of CRKP ST14 and ST2096 indicates successful adaptation and severe disease production capacity of these clones. High ceftazidime/avibactam resistance in MBL producer ST14 clone demonstrates an urgent need for new antibiotics that are active not only against OXA-48-like producers but also against MBL producers.

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