MOLECULAR ANALYSIS OF BETA LACTAMASES IN CLINICAL *ACINETOBACTER BAUMANNII* ISOLATES FROM INTENSIVE CARE UNITS

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ABSTRACT

MOLECULAR ANALYSIS OF BETA-LACTAMASES IN CLINICAL ACINETOBACTER BAUMANNII ISOLATES FROM INTENSIVE CARE UNITS

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Carbapenem resistance in *Acinetobacter baumannii* is a growing public health concern and represents a serious problem for treatment of the infection. Several carbapenem-hydrolysing β-lactamases have been identified from *A. baumannii* so far. In this study carbapenem resistance in *A.baumannii* strains recovered from intensive care units of Gulhane Military Medical Academy, Turkey, were investigated via multiplex PCR and with parallel phenotypic tests. From June 2006 to January 2010, 138 clinical *A. baumannii* isolates were collected. Identification and antimicrobial susceptibility tests of the isolates were performed. The MICs of imipenem and meropenem were determined by using E-test method. Carbapenem resistant A. baumannii strains were included for further study. Firstly, the presence of

carbapenemases were determined. The presence of Metallo-beta-lactamase (MBL) were also investigated. Detection of the four groups of OXA carbapenemases (OXA-23, OXA-24, OXA-51 and OXA-58) was carried out using a multiplex PCR assay. Sequence analyses were performed. Non-duplicate, multidrug resistant 61 clinical A. baumannii isolates were found to be resistant to imipenem and meropenem. In the 61 isolates, the MIC50 of imipenem and meropenem were 16 and >32; MIC90 were 192 and >32 respectively. Modified Hodge Tests (MHT) were positive for all 61 A. baumannii strains. None of these isolates showed MBL activity. As determined through multiplex PCR, all of the 61 isolates had $bla_{\text{OXA-51}}$ genes, 50 isolates had $bla_{\text{OXA-23}}$, and 11 isolated had $bla_{\text{OXA-58}}$ genes. Alleles encoding OXA-24-like enzymes were not detected in any isolates. This study indicated that the clinical isolates in our region contained $bla_{\text{OXA-51}}$, $bla_{\text{OXA-23}}$, and $bla_{\text{OXA-58}}$ resistance genes. However, $bla_{\text{OXA-24}}$ gene was either absent or occur in very low frequency.

Keywords: *Acinetobacter baumannii*, Carbapenemases, Oxacillinases

ÖZ

YOĞUN BAKIM ÜNİTELERİNDEN İZOLE EDİLEN KLİNİK *ACINETOBACTER BAUMANNII* İZOLATLARINDA BETA-LAKTAMAZLARIN MOLEKÜLER ANALİZİ

Üsküdar Güçlü, Aylin

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Acinetobacter baumannii izolatlarında karbapenem direnci artmakta olan bir halk sağlığı problemidir ve enfeksiyonların tedavisinde önemli bir sorun oluşturmaktadır. Bugüne kadar *A. baumannii* izolatlarında çeşitli karbapenem hidroliz eden β-laktamazlar tanımlanmıştır. Bu çalışmada Gülhane Askeri Tıp Akademisi yoğun bakım ünitelerinden izole edilen *A. baumannii* izolatlarında karbapenem direnci multipleks PCR ve paralel fenotipik testlerle araştırılmıştır. Mayıs 2006 Ocak 2010 yılları arasında 138 *A. baumannii* izolatı toplanmıştır. İzolatlar tür düzeyinde tanımlanmış ve antimikrobiyal duyarlılık testleri yapılmıştır. İmipenem ve meropenemin MİK değerleri E-test metodu ile saptanmıştır. Karbapenem dirençli

izolatlar takibeden çalişma için seçilmiştir. Öncelikle izolatlarda karbapenemaz varlığı gösterilmiştir. Metallo-beta-laktamazların (MBL) varlığı da araştırılmıştır. OXA carbapenemazların dört grubu (OXA-23, OXA-24, OXA-51 ve OXA-58) multiplex PCR yöntemiyle çalışılmıştır. Nükleotid dizi analizi yapılmıştır. Testlerden sonra saptanan 61 klinik izolatın hepsinin imipenem ve meropenem direnci gösterdiği bulundu. İmipenem ve meropenem için sırasıyla $MİK_{50}$ 16 and >32; $MİK_{90}$ ise 192 and >32 bulunmuştur. Modifiye Hodge Testi (MHT) 61 *A. baumannii* izolatının hepsinde pozitif olarak tespit edildi. İzolatların hiçbiri MBL aktivitesi göstermedi. 61 izolatın hepsi $bla_{\text{OXA-51}}$ geni; 50 izolatta $bla_{\text{OXA-23}}$ geni; 11 tanesindeyse $bla_{\text{OXA-58}}$ geni saptandı. OXA-24 enzimlerini kodlayan alellere hiçbir izolatta rastlanmadı. Bu çalışma, bölgemizdeki klinik izolatlarda $bla_{\text{OXA-51}}$, $bla_{\text{OXA-23,}}$ ve $bla_{\text{OXA-58}}$ direnç genlerinin bulunduğunu göstermiştir. Ancak, $bla_{\text{OXA-24}}$ geni ya hiç bulunmamakta ya da oldukça düşük frekansda bulunmaktadır.

Anahtar Kelimeler: *Acinetobacter baumannii,* Karbapenemazlar, Oksasilinazlar

To My Daughter Defne Beril ....

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TABLE OF CONTENTS

xi

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

AN	: Amikacin
CAZ	: Ceftazidime
CDT	: Combined disc test
CIP	: Ciprofloxacin
CLSI	: Performance Standards for Antimicrobial Susceptibility Testing
Co	: Colistin
CTX	: Cefotaxime
DDST	: Double Disc Synergy test
EMB	: Eozine Methylene Blue agar
FEB	: Cefepime
FOX	: Cefoxitin
GN	: Gentamicin
ICU	: Intensive Care Unit
IMP	: Imipenem
MBL	: Metallo-β-lactamase
MEM	: Meropenem
MHT	: Modified Hodge test
MIC	: Minimum Inhibitory Concentration
PRL	: Piperacillin
PTZ	: Piperacillin/tazobactam
PCR	: Polymerase chain reaction
SAM	: Ampicillin/sulbactam
SXT	: Trimethoprim/sulfamethoxazole

CHAPTER I

INTRODUCTION

The nonfermentative gram-negative bacilli are a group of aerobic, non-spore-forming bacteria. They do not use carbohydrates as a source of energy and do not degrade them through metabolic pathways other than fermentation (Koneman, 2006). *Acinetobacter* species became important human pathogens especially for last decade (Gillespie and Hawkey, 2006).

Acinetobacter species are saprophytic bacteria. They are found in nature and hospital environment (Forbes *et al.,* 2002; Gales *et al*., 2001). These species may become the member of skin and respiratory flora of patients hospitalized for prolonged periods. Hospital environment or colonised or infected patients and staff may become a source of these strains. Colonization of hospitalized patients by the bacteria may arise from environmental factors and medical instrumentation such as, intravenous or urinary catheters (Forbes *et al.,* 2002).

A nonfermenter isolate belong to the genus *Acinetobacter* has typical gram-stain morphology: gram-negative coccobacillary cells appearing as diplococci (figure1.1).

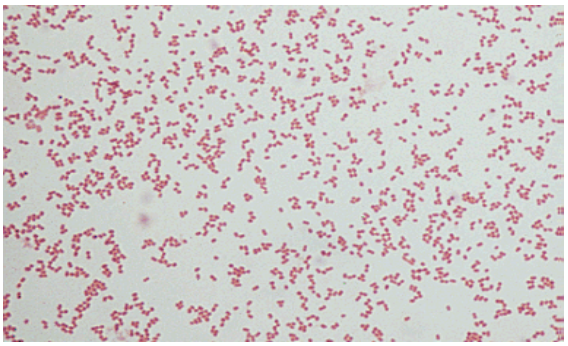

Figure 1.1. *Acinetobacter* image taken through light microscope (www.medic.med.uth.tmc.edu)

Acinetobacter species may be appeared as gram-positive coccobacilli in smears of positive cultures of blood samples and in smears of some other clinical specimens (figure1.2).

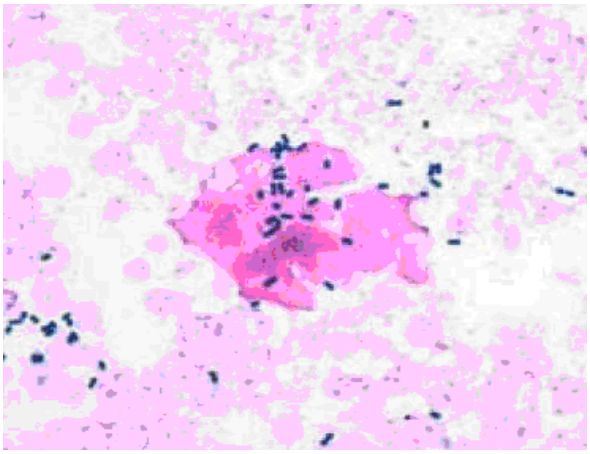

Figure 1.2. Acinetobacter staining gram-positive from positive blood culture (www.medscape.com)

After 24 hours of growth on blood agar, the colonies achieve between 0.5 and 2 mm in diameter. They seem as translucent to opaque, not pigmented, convex, and entire (figure 1.3). Most strains grow well on McConkey agar. Certain glucose-oxidizing acinetobacters may also cause a brown discoloration of tyrosine added heart-infusion agar and glucose included blood-agar. Presumptive identification of these strains can me made on the basis of the lack of cytochrome oxidase activity, lack of mobility, and resistance to penicillin (Koneman, 2006).

The *Acinetobacter* genus is currently classified in the *Moraxellaceae,* and this genus consists of 25 genomospecies. In human clinical samples, the most frequently recovered isolate is *A. baumannii* in the genus. *A. haemolyticus, A. lwoffii, A. johnsonii*, genomospecies 3, and genomospecies 6 are less common comparing with *A. baumannii*. *A. johnsonii*, *A. lwoffii,* and *A. radioresistens* are nonsaccharolytic *Acinetobacter* species that occur as natural inhabitants of human skin. *A. baumannii* is saccharolytic and produce acide from most of the carbohydrates. The absolute identification can be made by showing the rapid production of acid from lactose (Koneman, 2006).

Figure 1.3. *Acinetobacter* colonies on blood agar (http://thepetridishmicrobe.blogspot.com/**)**

A. baumannii is an important human pathogen. It is opportunistic bacteria. It has been reported in many infections last decades (Bergogne-Berezin and Towner, 1996). Pneumonia, urinary tract infections (UTIs), meningitis, peritonitis, endocarditis, wound infections are the examples of *A. baumannii* infections (Koneman, 2006). Outbreaks of hospital-acquired infections caused by *A. baumannii* isolates has been become a major problem particularly in intensive care units. Many reports of outbreaks related with *A. baumannii* infections have been published frequently (Bergogne-Berezin and Towner, 1996; Queenan and Bush, 2007; Liu *et.al.,* 2006).

1.1. Definition for *A. baumannii*

A. baumannii strains are strictly aerobic. Morphologically, they appear as diplococci or chains in Gram-stain examination. Actually the cells have polar fimbriae and they display 'twitching motility'. But, *A. baumannii* strains are accepted as non-motile. "ακινετοζ" the Greek word gives Acinetobacters' scientific name meaning 'not able to move'. All common media can be used to obtain *A. baumannii* colonies. Temperatures for incubation range from 20 to 30°C, but the optimal range is between 33–35 °C. Only few species can grow at 41 and 44°C. A Gram-negative bacillus can be considered as *Acinetobacter spp.,* if it has catalase activity, but not oxidase activity; and it is not able to use nitrate as a source of energy, and if it does not produce indole from tryptophan. For definition of strains *var. anitratus* or *var. lwoffii,* some biochemical characteristics including acid production from L-arabinose and D-glucose -ribose and -xylose can be used. All commercial automated identification systems use such phenotypic characteristics for identification of the *Acinetobacter* species (Koneman, 2006). More definite identification requires DNA-based methods (Gillespie and Hawkey, 2006).

1.1.1. Clinical Features of *Acinetobacter* Infections

Many nosocomial infections caused by *Acinetobacter* strains have been reported worldvide. Time and epidemiological factors efect the sites of *Acinetobacter* nosocomial infections. In the past, UTIs caused by *Acinetobacter spp.* were the most common infection in ICUs, but lately the incidence of UTIs is not so high. This may have been resulted from a more careful usage of the urinary catheters. Currently, hospital-acquired pneumonia caused by *Acinetobacter* strains has become more frequent than UTIs.

The incidence of *Acinetobacter* species in nosocomial pneumonia has been reported as 10% by a survey carried on Europe dealing with causative pathogens (Bergogne-Berezin, 2001). Surgical wound infections, endocarditis, meningitis, skin infections and peritonitis are less common and they are usually appeared as sporadic

cases (Bergogne-Berezin and Towner, 1996). Superinfection in burn patients is another important problem caused by *Acinetobacter* species. Severely burned patients have a risk for infections with multidrug-resistant (MDR) gram-negative bacillus including *Acinetobacter* species (Ferreira *et al.,* 2004).

1.2. Laboratory Diagnosis

1.2.1. Isolation

Common laboratory media are sufficient for the recovery of the frequently observed species of *Acinetobacter*. For the outbreak investigation, cystine-lactose-electrolyte-deficient agar or MacConkey agar can be used as selective or differential media. By the addition of antibiotics, these media can be made more selective for *Acinetobacter* strains with definite antimicrobial resistance patterns. *Acinetobacter* strains can be isolated specifically by using some more selective media; such as, Leeds *Acinetobacter* Medium (Koch, 2006). When studying with the environmental specimens, because of the small numbers of organisms, liquid enrichment will be useful (Bergogne-Berezin and Towner, 1996).

1.2.2. Identification

Even though some level of differentiation is possible, phenotypic characteristics are not always enough for the discrimination of the different genomospecies. *A. baumannii,* Genomospecies 2, is the most prevalent recovered strains in clinical specimens. Less majority of clinical isolates belong to *A. lwoffii A. johnsonii* and *A. junii.* For the identification of *Acinetobacter* species, many laboratories use commercial phenotypic systems. Molecular identification of genomospecies can be performed by only few clinical laboratories because of the high cost and the need of trained staff. Among molecular techniques, ribotyping and RNA sequence fingerprinting have been shown to accurately discriminate genomospecies (Koch, 2006).

1.2.3. Strain Typing

To prevent the spread of infections, the source and the way of spread of strains should be determined since outbreaks of *Acinetobacter* nosocomial infections have become a major problem in intensive care units. Many typing methods including bacteriocin or phage typing can be used for this purpose. PFGE can be performed to analyse restriction length polymorphisms in chromosomal DNA. PCR fingerprinting, ribotyping and random amplified polymorphic DNA analysis (RAPD) as well as AFLP fingerprinting techniques are more reliable molecular methods in strain typing. Although there is not a standard method for exact differentiation between clinical isolates, these methods can be used safely. Not many clinical laboratories have the facilities to use these methods. Antibiogram and biotyping methods are used instead of molecular techniques. To detect the relation between the pathogens, determining and improving the typing method is very important during endemic *Acinetobacter* infection or outbreaks (Koch, 2006).

Intrinsic resistance to more than one group of antimicrobial agent and their high tendency to gain antimicrobial resistance is a very important problem in *Acinetobacter* spp. Antimicrobial susceptibility rates in *Acinetobacter* strains recovered from clinical samples have been decreasing gradually since 1975. There have been many surveillance studies emphasizing the high resistance rates in clinical *Acinetobacter* strains. Aminoglycosides, monobactams, cephalosporins, extended-spectrum penicillins, fluoroquinolones have not been as effective as before (Gales *et al*., 2001). Susceptibility rates to CIP, GN, PRL and CAZ have been stated as less than 50% by a European study performed in ICUs. Five countries were included this study. Resistance patterns of the isolates show differences among countries since, different strategies are followed in the use of antibiotics connected with species distribution. Another study from United Kingdom dealing with 595 *Acinetobacter* isolates, pointed out that ceftazidime resistance rate was 89%, gentamicin and ciprofloxacin resistance rates were 40% among *A. baumannii* (Henwood *et al*., 2002). In treatment of *Acinetobacter* infections carbapenems are the most active antimicrobial agents. However, recent researches have pointed out the high level of

imipenem and meropenem resistance. In some studies, *A. baumannii* strains recovered from outbreaks of infection have been found as susceptible to only tigecycline or colistin antibiotics (Queenan and Bush, 2007).

Other *Acinetobacter* species including *A. lwoffii*, *A. junii* and *A. johnsonii* are less commonly seen in hospital-acquired infections and are usually found as susceptible to many antimicrobial agents. However, some studies pointed out the increasing resistance in other *Acinetobacter* species "(Bergogne-Berezin and Towner, 1996)".

1.3.Bacterial Resistance

Mechanism of bacterial resistance to antibiotics is complex, varied and not completelly understood. Some of the important variables needed to understand resistance mechanisms are shown in Table 1.1.

Table 1.1. Factors in the Transfer and Expression of Bacterial Resistance (Koneman, 2006)

Characteristic	Variable	Comments
Location	Chromosomal	Genetic stability; expression usually constitutive
	Extrachromosomal	Plasmids easily mobilized, transferred cell to cell
	Transposon	move genetic material between chromosome and plasmid or between bacterial cells
Transfer	Conjugation	Either plasmid (R-factor) or transposon
	Transduction	Transfer by bacteiophage
	Transformation	Direct transfer of DNA from environment between compatible species
Expression	Constitutive	Produced with or without exposure to a stimulus
	Inducible	produced only after exposure to a stimulus
	Constitutive-inducible	produced at low level without stimulus;production greatly increased after stimulation

The genes for the resistance mechanism might be placed on the chromosome or on plasmid. Chromosomal DNA is stable, whereas transfer of plasmid DNA is easy between strains, species and even genus (Koneman, 2006).

1.3.1. Resistance to β –Lactams

Bacterial cell wall, also known as the peptidoglycan, or murein layer, have an very important role in bacterial cell life. Lack of a similar structure in human cells, makes the cell-wall the focus of attention for the development of bactericidal agents that are relatively nontoxic for humans (Forbes *et. al*., 2002).

Beta-lactam antimicrobial agents contain the four-membered, nitrogen-containing, beta-lactam ring at the core of their structure (Figure1.4). Commonly used beta-lactams are Penicillins (penicillin, ampicillin, piperacillin, and mezlocillin), Cephalosporins (cefazolin, cefuroxime, cefotetan, cefotaxime, ceftriaxone, ceftazidime, and cefepime), Monobactams (aztreonam) and Carbapenems (imipenem, meropenem) (Figure 1.5). This drug class comprises the largest group of antibacterial agents, and dozens of derivatives are available for clinical use (Forbes *et al.*,2002).

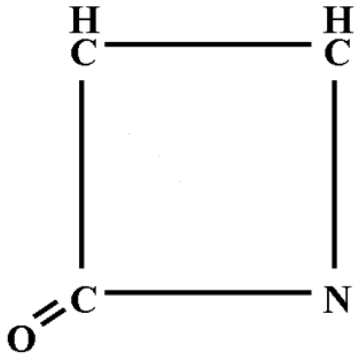

Figure 1.4. Beta-lactam ring (http://www.life.umd.edu/)

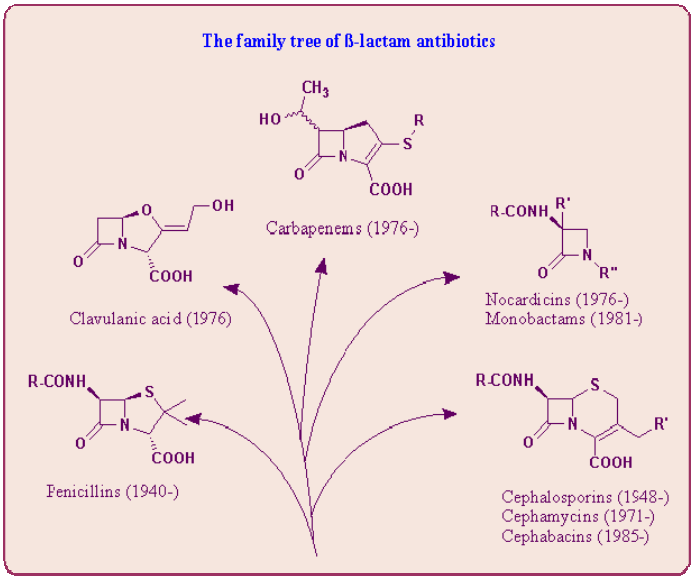

Figure 1.5. The family members of β-lactams (http://www.cic.klte.hu/)

The β-lactam ring is the key to mode of action of these drugs. The β-lactam antibiotics inhibit synthesis of cell wall. The enzymes included in cell wall synthesis are bound by β-lactam ring. Cell wall synthesis is stopped. When the bacterial cell lost the capacity to synthesize and preserve its peptidoglycan structure, it cannot survive. Because of the osmotic instability caused by faulty cell wall synthesis, death occurs, or the binding of the β-lactam to PBP may cause a series of events leading to autolysis and death of the cell. These enzymes required for this function are anchored in the cell membrane and as groups are refferred to as penicillin-binding protein, or PBPs. Bacterial species may contain between four to six different types of these binding proteins. These transpeptidases have the most essential role for survival (Forbes *et al.*,2002). The β-lactam type antibiotics inactivate these transpeptidases (Koch, 2006). Because all clinically relevant bacteria have cell walls, β-lactam agents act against a broad spectrum of bacteria (Forbes *et al.*,2002).

Enzymatic inactivation especially β-Lactamase production and altered receptors are common resistance mechanisms which act on β–Lactams. *Acinetobacter* species contain different genes encoding various β-Lactamase enzymes (Koneman, 2006).

1.3.2. Resistance to Aminoglycosides

Aminoglycosides are protein synthesis inhibitor. They act on the 30S subunits of rybosome. They separate peptide chains from 30S subunit and protein synthesis stop. They are used especially on the treatment of infections with gram-negative bacillus. The most effective aminoglycoside is amikacin (Murrey *et al.,* 2010). Aminoglycoside modifying enzymes can inactivate aminoglycosides. There is a wide variaty of such enzymes. Even amikacin as the most effective agent against *Acinetobacter,* can escape completelly from enzymatic neutralization (Koch, 2006).

1.3.3. Resistance to Fluoroquinolones

They are synthetic antibacterial compounds. They act on the bacteria by binding the α-subunit of DNA gyrase. They prevent the gyrase from supercoiling bacterial DNA. Fluoroquinolones are broad spectrum antimicrobial agents (Murrey *et.al.,* 2010). They were highly active against *Acinetobacter* when they were first introduced, hovewer, high incidence of fluoroquinolone resistance have been emphasised recently. Resistance is mainly mediated by the mutations in the genes reffered as *gryA* and *parC* (Koch, 2006).

1.3.4. Resistance to Other Antibiotics

Tetracyclines are agents that inhibit protein synthesis. They act on all bacterial strain except *Staphylococcus, Enterococcus* and *Acmetobacter spp*. (Murray, *et.al.,* 2010). Resistance to tetracycline in *A. baumannii* is resulted from *Tet*A and *Tet*B efflux proteins (Koch, 2006). Sulfametoksazol and trimetoprim inhibit the two enzymes which have a consecutive role in tetrahydropholate sysnthesis (Murray, *et.al.,* 2010). Trimethoprim and chloramphenicol show mainly plasmid-mediated resistance mechanism (Bergogne-Berezin and Towner, 1996).

1.4. Treatment of *Acinetobacter* Infections

High levels of antimicrobial resistance to multiple groups of agents restrict the therapeutic choises for the treatment of nosocomial *Acinetobacter* infections (Koch, 2006). For empirical treatment of nosocomial pneumonia, studies with animal models of *Acinetobacter* pneumonia have guided to use carbapenems or carboxypenicillins mostly combination with an aminoglycoside. The use of β-lactamase inhibitors can be another strategy for treatment. Among β-lactamase inhibitors, sulbactam can be used as a single drug in the treatment. Efficiency of sulbactam is high because of its intrinsic activity. Sulbactam have no influence on β-lactam activity (Karageorgopoulos and Falagas, 2008). This agent also has better activity than clavulanic acid and tazobactam (Koch, 2006).

1.5. Carbapenem Resistance

Imipenem and meropenem as carbapenem are active against *Acinetobacter* strains. Among all β-Lactams imipenem and meropenem have the broadest spectrum on bacterial pathogens. Carbapenems have not effect on cell-wall-deficient organism like *Mycoplasma*, mycobacteria, and several non-fermenters (Livermore and Woodford, 2000). They are used to treat a variety of serious infections when an organism is resistant to the primary agent of choise. Carbapenems are also used to treat nosocomial and mixed bacterial infections (www.cdc.gov.tr). They have not lost their activity against ESBL (+) Gram-negative bacteria and against bacteria which are hyperproducer of AmpC β-Lactamases. These β-Lactamases are important widespread enzymes that cause resistance to some cephalosporins (Livermore and Woodford, 2000).

Some β-Lactamases that can destroy imipenem and meropenem have been identified. Beside enzymatic inactivation, other mechanisms have also been described. Carbapenem resistance may be particularly due to the loss of outer membrane proteins (Bou *et al*., 2000).

1.5.1. Carbapenem Resistance Mechanisms in *Acinetobacter baumannii*

Carbapenems including imipenem and meropenem as the broadest spectrum β-lactam antibiotics were introduced in clinical use in 1985. They are the most effective agents. For treatment of multidrug-resistant *A. baumannii* infections imipenem is commonly used because of its efficiency. But recently, resistance in *Acinetobacter* strains to carbapenem has been reported frequently. *Acinetobacter* strains showed high level of carbapenem resistance in many researches. One of the major problems in ICUs is carbapenem resistance in *Acinetobacter* (Zarilli *et.al*, 2009).

A. baumannii have varied types of resistance mechanisms to all currently used antibiotic classes. *A. baumannii* strains also have ability to acquire new reistancse determinants. Some resistance islands have been detected by genome sequence analysis of clinical MDR *A. baumannii* strains. These islands had various arrangements of resistance genes carrying transposons, integrons, and some other mobile genetic elements. It is known that resistance genes carrying plasmids are present in *A. baumannii* strains. These genes are involved in Horizontal gene transfer (Roberts MC, 2006; Zarilli *et al*., 2009).

1.5.2. Sources of carbapenem resistance in *A.baumannii*

1.5.2.1. β –lactamases

Penicillins, carbapenems, monobactams, and cephalosporins are the members of β-lactams and can be inactivated by hydrolysis by β-lactamases. Enzymatic inactivation is one of the most common resistance mechanisms. Heavy pressure of antibiotic use may cause mutation on genes coding for β-lactamase enzymes leading to development of newer broad spectrum β-lactamases (Noyal *et al*., 2009).

- **OXA-type Carbapenemases:** Eight different families constitute OXA-type carbapenemases in *Acinetobacter* (table 1.2). The sequence similarities within

each family are more than 92.5 per cent. Between OXA-types that belong to different cluster, the identities may range from 40 to 70 per cent. Clinical *A. baumannii* strains have mainly four clusters. The first group is OXA-23, named ARI-1 comprised from OXA-23, -27 and -49. Two to five amino acids may differ between these types. The second group represented by OXA-24, contains the OXA-24, -25, -26, -40 and -72 β-lactamases. 1 to 5 amino acids may differ between these types. The third family includes intrinsic OXA-51 β-lactamase variants (Walter-Rasmussen and Hoiby, 2006). All of the *A.baumannii* isolates carry these OXA-51 genes on their chromosomes (Poirel and Nordman, 2006). Different OXA-51 variants have been defined in *A.baumannii* strains from different regions. The newly identified OXA-51 group contains OXA-64-66, OXA-68-71 and OXA-75-78 (Brown *et.al*, 2005), OXA-88, OXA-91, OXA-93, OXA-94 and OXA-95 (Koh *et.al.,* 2007). The fourth group is OXA-58 which has only a single member (Walter-Rasmussen and Hoiby, 2006).

Table 1.2.OXA-type carbapenemases

Enzyme Subfamily	**OXA members**
OXA-23 (ARI1)	OXA-23 OXA-27 OXA-49
OXA-24	OXA-24 OXA-25 OXA-26 OXA-40 OXA-72
OXA-51	OXA-64-71 OXA-75-78 OXA-83-84 OXA-86-89 OXA-91-92 OXA-94-95
OXA-58	OXA-58

- **Metallo-β-Lactamases:** Carbapenem resistance in *A. baumannii* may also arise from enzymes called metallo-β-lactamases (MBLs). MBLs have the ability to hydrolyse lots of antibiotic group including imipenem and meropenem. The dissemination of MBLs is often plasmid-mediated and rapid (Noyal *et al.,* 2009). These acquired enzymes exist in Ambler class B enzymes (Ambler, 1980). MBLs have been reported only rarely in clinical acinetobacters. MBLs are susceptible to EDTA inactivation. This characteristic can be used in laboratory detection of MBL production. E-test strips containing imipenem and imipenem with EDTA are very useful for detection of MBL production in laboratory conditions (Poirel *et.al*, 2006).

- **AmpC β-lactamases:** AmpC β-lactamases are encoded chromosomally or by plasmids and are found in *Enterobacteriaciae* and several other, gram-negative bacteria like *Pseudomonas* and *Acinetobacter*. AmpC enzymes belong to Ambler class C classification system (1980). AmpC encoded β-lactamases may confer clinically important β-lactam resistance and can interfere with detection of extended spectrum betalactamases (ESBLs) in case of AmpC overexpression. AmpC β-lactamase can be over-expressed or can be inducible upon exposure to β-lactam antibiotics. β-lactamase inhibitors of Ambler class A β-lactamases are mostly ineffective. If AmpC expression combined with outer membrane, porin loss increases carbapenem MICs sharply. AmpC β–lactamases combined with porin deficiency trigger carbapenem resistance in *A. baumannii* strains (Quale *et al.*, 2003).

1.5.2.2. Porin loss or modification

Carbapenem resistance may also arise from the loss of outer membrane proteins (OMPs) or from the mutation in porins. Mutations in porins lead the lack of drug penetration (Noyal *et.al.,* 2009). OMPs have a role in acquiring resistance to carbapenem in clinical strains of acinetobacters (Zarilli *et.al.,* 2009). The loss of related OMP (29-kDa) in the strains without carbapenem hydrolyzing enzyme

activity, confer imipenem resistance (Limansky *et al*., 2002). This heat-modifiable 29-kDa OMP, is renamed as CarO. This gene encodes a 247 amino acid polypeptide. Influx of imipenem and meropenem antimicrobials are controlled by *carO* gene in *A. baumannii* strains (Poirel *et al.*, 2006).

1.5.2.3. Modification of PBPs

Modification of PBPs is another reason of carbapenem resistance. This mechanism has been studied only in a few researches. Hyper production of 24-kDa PBP and low level of six other PBPs have been detected in resistant mutant strains of *A.baumannii* (Fernandez *et al.*, 2003). Missing of 73.2-kDa PBP with the production of carbapenemases cause carbapenem resistance in *A.baumannii* strains with >4 mg/L value of MIC (Poirel, 2006).

AdeABC efflux mechanism has been determined in *A. baumannii* strains. Efflux mechanism contains a wide range of structurally unrelated molecules (Noyal et al., 2009). Although some antimicrobial resistance may be gained by efflux pump system, efflux pump has not been detected as responsible mechanism for resistance to carbapenems (Quale *et al.*, 2003).

1.6. Screening Tests for Detection of Carbapenemases

There are different phenotypic methods to detect carbapenem activity. But, there still has been no standard guideline for screening of carbapenemases for a long time. Recently, CLSI has recommended modified Hodge test (MHT) for detection of carbapenemase activity. The MHT is useful in cases of infection control and epidemiological purposes (CLSI, 2008).

Various methods like EDTA disk synergy, MBL E-test, EDTA combined disk test are used for detection of MBLs. However, EDTA disk synergy test is very simple and sensitive technique to detect MBL activity (Noyal *et al.,* 2008).

Production of AmpC β-lactamase can be detected by a test that uses boronic acid. If the boronic acid increases the inhibition zones to 5 mm or more, AmpC is

present. AmpC disk test and three-dimentional extract method are the frequently used tests for screening the presence of AmpC enzymes (Noyal *et al.*, 2008).

PCR technique has been commonly preferred to detect the genes encoding carbapenem resistance because it's reliable and satisfactory results.

1.7. Aim and Scope

There is a special requirement to determine the source of carbapenem resistance in *A. baumannii* to prevent current therapeutic failures. All alternative empirical treatment strategy for infections with *A. baumannii* strains resistant to imipenem and meropenem result in failure. Dissemination of MDR strains and therapeutic failures have become the major problem to solve. Future research should include the development of new inhibitors of β-lactamases which inactivate carbapenems. This study aimed to research the role of β-lactamases in carbapenem resistance in *A. baumannii* strains of intensive care units of Gulhane Military Medical Academy, Ankara, Turkey. The study was conducted by using DNA-based molecular typing of MDR *A. baumannii* isolates in an attempt to characterize the cause of antimicrobial resistance which will help to control the spread of *A. baumannii* strains causing infections from one geographic region to the others.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

Table 2.1 Chemicals and Suppliers

Chemicals	**Suppliers**
Agarose	Sigma
Dimethylsulfoxide	Sigma
DNA Size Marker	Fermentas
dNTP	Amresco
EDTA	Sigma
Ethidium Bromide	Amresco
Ethanol	Merck
Isopropylalcohol	Merck
Orange G	Sigma
Phenol:Chloroform:Isoamyl Alcohol 1	Amresco
Phase	Sigma-Aldrich
Phenylboric acid	Sigma
Primers	Amresco
Proteinase K	Applied Biosystems, Roche
Taq DNA Polymerase	BioRon

Table 2.2 E-test strips, Discs and Suppliers

E-test strips and Discs	**Suppliers**
E-test Imipenem	AB Biodisk
E-test Meropenem	AB Biodisk
E-test MBL	BioMérieux
Blank Discs	Oxoid
10µg İmipenem disc	Oxoid
10µg Meropenem disc	Oxoid
Cefotetan disc	Oxoid
Imipenem disc	Oxoid
Meropenem disc	Oxoid

Table 2.3 Media and Suppliers

Media	**Suppliers**
EMB agar	Salubris
%5 Sheep Blood agar	Salubris
Muller Hinton agar	Salubris

Table 2.4 Equipment and Supliers

Equipment	**Suppliers**
Dry Block Heating Thermostat	Boeco
PCR system	Applied Biosystems,
Incubator EN500	Nüve
McFarland Reader	BD
Centrifuge 5415R	Eppendorf
Electrophoresis	BioRad
Vortex	Velp
Phoenix ^{TM} 100 instrument	Becton Dickinson
Phoenix NMIC/ID panel	Becton Dickinson
Power Supply	BioRad
Gel Doc Transilluminator	BioRad

Table 2.5 ATCC Control Strains

Strain	ATCC Number
Escherichia coli	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Stenotrophomonas maltophila	ATCC 13636

2.2.Methods

2.2.1. Sampling of the isolates

From June 2006 to January 2010, non-duplicate clinical isolates of *A. baumannii* were collected from Intensive Care Units (ICU) of Gulhane Militay Medical Academy. The isolates were categorized as imipenem and meropenem resistant according to CLSI recomendation. Isolates were stored at -80°C in 20% skim milk solution until use.

2.2.2. Culture

All clinical specimens sent for bacteriological investigation were cultured by standard microbiologic techniques (Koneman, 2006). Specimens were incubated in 5% CO_2 incubator for 24 hours on Eozine Methylen Blue (EMB) agar, and 5% sheep blood agar plates.

2.2.3. Identification

Organisms were identified by standard microbiological methods (Koneman, 2006). After 24 hours of growth on blood agar, the colonies between 0.5 and 2mm in diameter, opaque, and convex were considered as *Acinetobacter*. These strains appeared as gram-negative coccobacillary cells, often diplococci on Gram-stain. Oxidase activity negative, nonmotile, and penicillin resistant non-fermentative aerobic Gram-negative coccobacilli were considered as *Acinetobacter spp*.

To identify the microorganisms, NMIC/ID panel of Phoenix^{TM} Automated Microbiology system was used according to the direction of Becton Dickinson Diagnosis.

2.2.4. Antimicrobial Susceptibility by Phoenix System

The MIC values of antimicrobial agents were determined by using NMIC/ID panel of Phoenix^{TM} 100 instrument,. The instrument incorporates the use of an oxidation-reduction indicator, turbidometric growth detection, "full on-panel" antimicrobial concentrations. Its software gives the MIC values and interpretation of the final results acording to the CLSI.

2.2.5. Definition of multidrug-resistant (MDR) strains

Although there are different definitions of MDR, *A. baumannii* isolates were defined as MDR, when they were resistant to at least three representative antimicrobial agents of different classes of antibiotics. Carbapenems (imipenem or meropenem), aminoglycosides, antipseudomonal penicillins, 3$^{th.}$generation cephalosporins and quinolones are frequently tested agents to detect resistance. Besides these agents, colistin, ampicillin/sulbactam and tetracyclines are tested occasionally (Falagas *et al.,* 2006). Accordingly, amikacin (AN), ampicillin/sulbactam (SAM), cefepime (FEB), cefotaxime (CTX), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN), piperacillin (PRL), piperacillin/tazobactam (PTZ), trimethoprim/sulfamethoxazole (SXT), and colistin (Co) were tested in this study.

2.2.6. E-test for Imipenem and Meropenem Susceptibility

MDR strains of *Acinetobacter baumannii* determined by Phoenix^{TM} system were subjected to the E-test method, a quantitative technique to determine imipenem and

meropenem MIC values. The suspention of isolated colonies and agar plates were prepared as described below.

- After inoculating the test organism, E-test strips were put on MHA surfaces with a sterile forceps. The strips were positioned with the MIC scale facing upwards.
- After 15 minutes, inverted plates were incubated at 35°C
- After 16 to 20 hours, each plate was carefully read.
- MIC values were recorded where the edge of the inhibition ellipse intersects the numbers on the strip.

2.2.6.1. MIC Interpretive Standards For *Acinetobacter* spp.

MIC interpretive standars of CLSI (2010) were used for interpreting E-test MIC values. Microcolonies and hazes were considered as growth to detect the point of whole inhibition. MICs were tabulated for each

The endpoints were examined carefully. The zone margins with area showing no obvious, visible growth were measured.

CLSI gives the MIC interprevative standard (μg/mL) for both imipenem and meropenem; ≤ 4 as sensitive (S), 8 as intermediate (I), and ≥16 as resistant (R). Table 2B-2 (Zone Diameter and MIC Interpretative Standards for *Acinetobacter spp.*) of the CLSI M100-S20 (2010), yearly revised international manual.

2.2.7.Screening Tests for Detection of Carbapenemases

2.2.7.1. Modified Hodge Test

Modified Hodge Test (MHT) was applied by following CLSI recommendation (2008). The imipenem and meropenem resistant strains were tested to detecet their carbapenemaseactivity by MHT.

- ATCC 25922 *Escherichia coli* was the indicator organism and 0.5 McFarland of suspension was prepared from overnight culture.

- By using a sterile cotton swab this suspention was applied on the surface of plate. At this step Mueller-Hinton agar (MHA) was used.
- Before incubation, inoculated plate was waited for 15 min at room temperature,
- At the center of the plate, 10-µg imipenem disc was placed.
- Test organism was streaked from imipenem disk to the edge of the plate. This step was repeated in four different directions.
- The plate was allowed for incubation at 37ºC for 18-24 hours.
- Production of carbapenemase was observed by distorted inhibition zone around imipenem disk. Clover leaf shaped zone was considered as positive result.

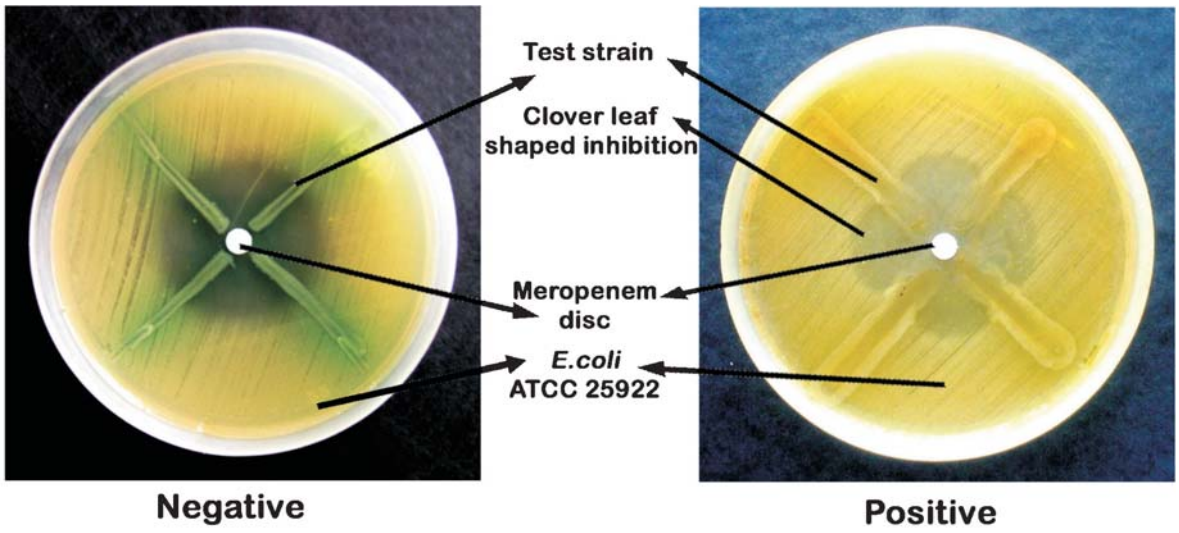

Figure 2.1. Modified Hodge Test. Cloverleaf shaped zone of inhibition indicates the positive result. Negative strain shows an undistorted zone of inhibition (Koch, 2006).

2.2.7.2. Imipenem-EDTA combined disc test (CDT)

CDT was performed to detect metallo-β-lactamases (MBLs) production (Yong *et al.* 2002).

- 186.1g of disodium EDTA was dissolved in 1L of distilled water. EDTA solution was 0.5M. (*p*H was 8.0)

- 0.5 McFarland suspension of the test isolate from overnight culture was prepared.
- Using a sterile cotton swab this suspention was spread on the surface of a MHA plate.
- Two 10 µg imipenem disks were placed on the plate
- 10 µL of EDTA solution was added to one of them.
- After 16 to 18 hours of incubation at 35°C, the inhibition zones of the imipenem and imipenem-EDTA disks were compared.
- The ≥ 7 mm increase in inhibition zone with the imipenem and EDTA discs was considered as positive MBL production (Figure 2.2).

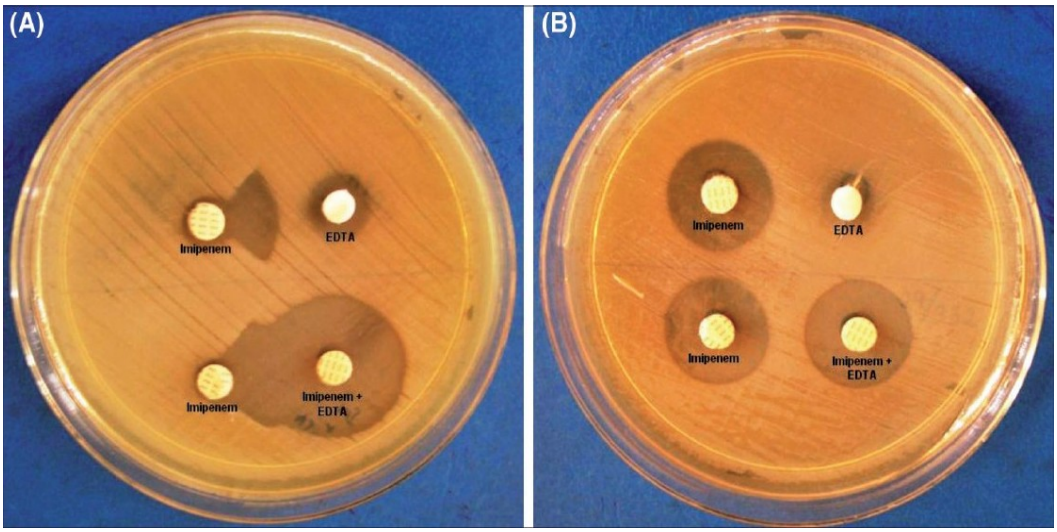

Figure 2.2: DDST (Upper half) and CDT. (A) MBL positive (B) MBL negative (Koch, 2006).

2.2.7.3. Imipenem-EDTA double disc synergy test (DDST)

DDST test was performed according to the direction defined previously (Lee *et al.,* 2003).

- Overnigth incubation of test organism was used to prepare 0.5 McFarland suspention and using a sterile cotton swab it was inoculated on MHA plates.

- 10 µL of 0.5 M EDTA solution was added to blanck disk. Imipenem disc was placed 20 mm apart from blank disc (from center to center).
- Enhancement of the inhibition zone in the region between imipenem and the EDTA disc was considered as a positive result (Figure 2.2)

2.2.7.4. Metallo-β-Lactamase E-test

MBL E-test strip consisting of imipenem (IP)/ imipenem + EDTA (IPI) was used to detect MBLs. The test was set up using a standard E-test procedure as discribed before. ATCC 27853 *P. aeruginosa* was used as negative and *S. maltophilia* ATCC 13636 as positive control.

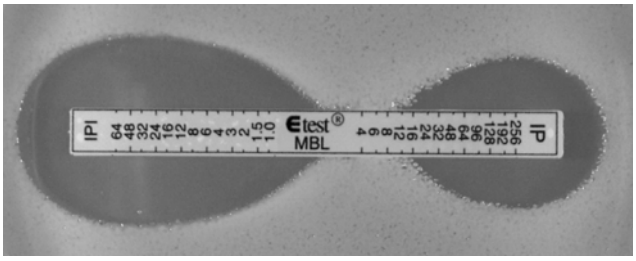

Figure 2.3.a Clear cut MBL positive: MIC IP/IPI= 16/<1 = >16 (test kit manuel, ABbiodisk)

Figure 2.3.b. Phantom zone (test kit manuel, ABbiodisk)

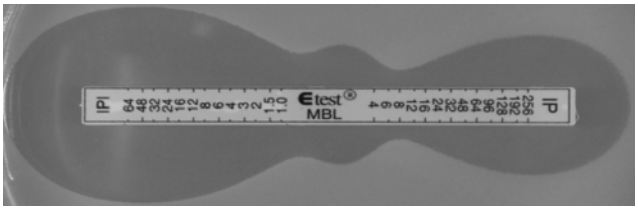

Figure 2.3.c. Deformation of the IP or IPI ellipse is indicative of MBL (test kit manuel, ABbiodisk)

When bacterial growth was clearly visible after 16-18 hours incubation, the IP and IPI MIC values were recorded and ratio of IP/IPI MIC value was calculated. ≥8 or ≥3 $\log_2$ dilutions in the IP/API value indicated the production of MBL enzyme (figure 2.3). Phantom zone and ellipse zone deformation around the strip were also accepted as positive without calculating the ratio (figure 2.3.b).

2.2.7.5. Boronic acid (BA) disk test for Amp C β-Lactamase

Boronic acid disks were prepared as follow (*Coudron,* 2005).

- 3 ml of dimethylsulfoxide was used to dissolve 120 mg of phenylboronic acid.
- Three milliliters of sterile distilled water was added.
- 20 µL from this prepared solution was inoculated onto disks containing 30 µg of cefoxitin (FOX) and onto blank disk.
- Disks were dried for 30 min at 70°C.
- They were stored in airtight vials at 4°C until use.
- Test organism (overnight culture) was adjusted to 0.5 McFarland turbidity
- these suspention was inoculated on MHA by usinf a cotton swab
- disk containing 30 µg FOX and 400 µg of BA of was placed onto agar.
- Plates were incubated at 35°C for 18-24 hours.
- 5mm diameter enhancement of the zone around the disk containing FOX and BA compared the zone around FOX disk considered as positive (figure 2.4).

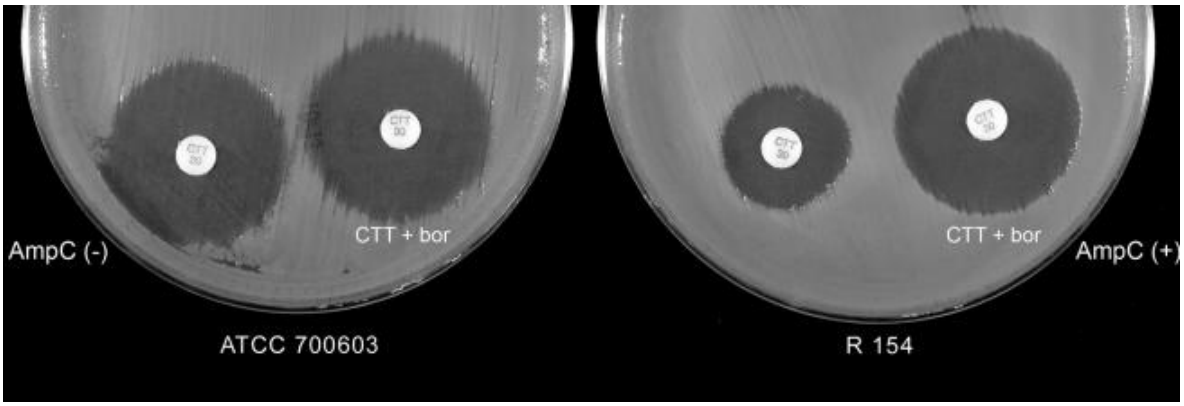

Figure 2.4. Boronic acid disk test. AmpC β-lactamase positive for Strain ATCC 700603 and negative for R154 (*Coudron,* 2005)

2.2.8. Detection of Oxacillinases by Multiplex PCR

The bla_{OXA} alleles encoding carbapenem hydrolyzing OXA enzymes were seached in 61 clinical *A. baumannii* isolates by using a multiplex PCR method (Woodford *et al*., 2006).

2.2.8.1 Primers

1. OXA-51-like amplifying 353 bp PCR product
    - **5'-TAA TGC TTT GAT CGG CCT TG**
    - **5'-TGG ATT GCA CTT CAT CTT GG**

Primer pair given above amplifies a fragment of 353 bp on gene encoding OXA-51 enzymes in *A. baumannii*. This gene is intrinsically located on chromosomes of the strain. This primer pair was used together with following primers previously designed by Woodford *et.al.* (2006) to amplify fragments of genes encoding acquired OXA-23, OXA-58 and OXA-24 carbapenemases given below;

2. OXA-23-like amplifying 501 bp PCR product
    - **5'-GAT CGG ATT GGA GAA CCA GA**
    - **5'-ATT TCT GAC CGC ATT TCC AT**

3. OXA-24-like amplifying 246 bp PCR product
    - **5'-GGT TAG TTG GCC CCC TTA AA**
    - **5'-AGT TGA GCG AAA AGG GGA TT**

4. OXA-58-like amplifying 599 bp PCR product
    - **5'-AAG TAT TGG GGC TTG TGC TG**
    - **5'-CCC CTC TGC GCT CTA CAT AC**

2.2.8.2. DNA Extraction

DNA was extracted from fresh culture of *A. baumannii* colonies according to the following protocol.

1. 15 µl Proteinase K (20mg/ml)
   50 µl SDS (10%)
   450 µl K Buffer
   A loop of bacterial colony
   were added into a sterile eppendorf tubes, and were incubated 1 hour at 55°C.
2. By adding 500 µl of Phenol:Chloroform:Isoamylalcohol (25:24:1), tubes were centrifugated at 12.000 rpm for 5 minutes.
3. A new sterile eppendorf was used to transfer the upper.
4. 500 µl isopropile alcohol was added and vortexed. Mixture was centrifugated at 12.000 rpm for 10 minutes.
5. The supernatant was removed.
6. 500 µl ethyl alcohol was added and vortexed. Mixture was centrifugated at 12.000 rpm for 10 minutes.
7. The supernatant was removed, and tubes were placed into 50°C incubator for drying.
8. After drying, 200 µl of sterile distilled water was added.
9. Extracts were stored at -20°C until use.

2.2.8.3. PCR Mixture

- 10X PCR buffer
- 0.2 mM dNTP
- 4mM $MgCl_2$
- 0.5 µl from each primer (50 pmol/ml)
- 0.2 µl of *Taq* DNA polymerase (5U/ µl)
- 4.8 µl dH_2O

3 µl template DNA was added to 47 µl of PCR mixture competing total volume to 50 µl.

2.2.8.4. Amplification

The conditions of amplification;

- initial denaturation: 94°C for 5 min
- 30 cycles; 94°C for 25s, 52°C for 40s and 72°C for 50s
- final elongation: 72°C for 6 min

2.2.8.5. Analysis of PCR Products

Electrophoresis was applied for the analysis of the amplicons. Agarose gel (2%) was prepared and ethidium bromide was added. PCR products were visualized over UV illuminator.

- 0.6 mg agarose was added in 30 ml TBE buffer, and this solution was boiled.
- Before transfer into the electrophoresis tank, 5 µl ethidium bromide was added into the boiled gel.
- 10 µl of amplicon was mixed with 2-3 µl of Orange G (loading buffer). From this mixture 10 µl was loaded to the well of agarose gel electrophoresis.
- DNA size marker was added to first well.
- Power supply was adjusted to 100 volt for 25 minutes, and gel was placed into the 0.5X TBE buffer in the tank.
- The amplicons were visualized over UV illuminator.

2.2.8.6. DNA Sequence Analysis

PCR product were analysed by RefGen BioTechnology.

CHAPTER III

RESULTS

During this study period, 138 *A. baumannii* isolates were recovered from ICUs of Gulhane Military Medical Academy. From these 138 isolates, imipenem and meropenem resistant 61 strains were selected for the further analysis. The minimum inhibitory consentration (MIC) of the antimicrobial agents, were given in Table 3.1. These 61 resistant *A. baumannii* isolates reflected the high resistance against other antimicrobial agents including; amikacin (AN), gentamicin (GN), ciprofloxacin (CIP), ampicillin/sulbactam (SAM), cefepime (FEB), cefotaxime (CTX), ceftazidime (CAZ), piperacillin (PRL), piperacillin/tazobactam (PTZ), and trimethoprim/sulfamethoxazole (SXT); but not resistance to colistin (Co) (Table 3.2).

All isolates were defined as multidrug-resistant *A. baumannii*. The 24 (39.3%) strains were resistant (R) to all tested antibiotics tested except colistin. Sixteen isolates (26.2%) were intermediatelly susceptible (I) to only one antimicrobial agent (7 strains to SAM; 5 to FEB; 3 to CIP and 1 to GN). Twenty isolates (32.8%) were susceptible (S) to only one antibiotic (6 strains to SAM; 6 to GN; 4 to AN; 4 to CAZ) besides colistin.

Table 3.1 Antimicrobial Susceptibility Test, MIC values* (μg/L) of the *A. baumannii* isolates

NO	AN	SAM	FEB	CTX	CAZ	CIP	GN	IMP	MEM	PRL	PTZ	SXT
1	>32	16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
2	>32	16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
3	>32	8/4	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
4	>32	8/4	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
5	>32	16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76

Table 3.1 Cont.

NO	AN	SAM	FEB	CTX	CAZ	CIP	GN	IMP	MEM	PRL	PTZ	SXT
6	<=8	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
7	>32	8/4	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
8	>32	>16/8	>16	>32	>16	2	>8	>8	>8	>64	>64/4	>4/76
9	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
10	>32	16/8	>16	16	4	>2	>8	>8	>8	>64	>64/4	>4/76
11	>32	>16/8	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
12	>32	>16/8	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
13	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
14	>32	>16/8	>16	16	4	>2	>8	>8	>8	>64	>64/4	>4/76
15	>32	>16/8	>16	>32	>16	2	>8	>8	>8	>64	>64/4	>4/76
16	>32	>16/8	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
17	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
18	>32	>16/8	>16	>32	>16	>2	8	>8	>8	>64	>64/4	>4/76
19	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
20	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
21	>32	8/4	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
22	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
23	>32	>16/8	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
24	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
25	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
26	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
27	>32	8/4	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
28	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
29	>32	>16/8	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
30	>32	8/4	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
31	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
32	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
33	>32	>16/8	>16	>32	>16	>2	<=2	>8	>8	>64	>64/4	>4/76
34	>32	>16/8	>16	>32	8	>2	<=2	>8	>8	>64	>64/4	>4/76
35	>32	16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
36	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
37	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
38	>32	>16/8	>16	16	4	>2	>8	>8	>8	>64	>64/4	>4/76
39	<=8	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
40	<=8	>16/8	16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
41	>32	16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76

Table 3.1 Cont.

NO	AN	SAM	FEB	CTX	CAZ	CIP	GN	IMP	MEM	PRL	PTZ	SXT
42	>32	16/8	16	8	4	>2	>8	>8	>8	>64	>64/4	>4/76
43	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
44	>32	16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
45	>32	16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
46	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	0.5/9.5
47	32	8/4	16	16	4	>2	>8	>8	>8	>64	>64/4	>4/76
48	>32	>16/8	>16	>32	>16	>2	<=2	>8	>8	>64	>64/4	>4/76
49	>32	>16/8	>16	>32	>16	>2	4	>8	>8	>64	>64/4	0.5/9.5
50	<=8	16/8	>16	>32	>16	>2	16	>8	>8	>64	>64/4	0.5/9.5
51	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
52	>32	>16/8	>16	>32	>16	>2	<=2	>8	>8	>64	>64/4	>4/76
53	<=8	16/8	>16	>32	>16	>2	4	>8	>8	>64	>64/4	0.5/9.5
54	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
55	>32	16/8	>16	16	8	>2	>8	>8	>8	>64	>64/4	>4/76
56	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
57	>32	16/8	>16	>32	>16	>2	4	>8	>8	>64	>64/4	>4/76
58	>32	16/8	>16	>32	>16	>2	4	>8	>8	>64	>64/4	>4/76
59	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76
60	>32	>16/8	>16	>32	>16	2	>8	>8	>8	>64	>64/4	>4/76
61	>32	>16/8	>16	>32	>16	>2	>8	>8	>8	>64	>64/4	>4/76

*** Values obtained through Phoenix™**

The most effective antimicrobial agent against *A. baumannii* was colistin. Although ampicillin/sulbactam, imipenem, meropenem, gentamicin, and ciprofloxacin are given group A antimicrobal agents against *A. baumannii* in CLSI (2010), their susceptibility rates were 11.4%, 0%, 0%, 13.1%, 0% respectively. Among group A antimicrobial agents gentamicin was the most effective agent with 13.1% sensitivity.

The MDR resistant *A. baumannii* strains were recovered mostly from ICU of burn unit (45.9%). Table 3.3 shows the distribution of the ICUs were given in. The mean age of patiens was calculated as 36.2 (AppendixA).

Table 3.2. Antimicrobial resistance rates of antimicrobial agents (n=61).

Agents	R*	I**	S***
AN	90.2%	1.6%	8.2%
SAM	65.7%	22.9%	11.4%
FEB	92%	18%	0
CTX	90.2%	8.1%	1.7%
CAZ	88.5%	0	11.5%
CIP	94.1%	4.9%	0
GN	83.7%	3.2%	13.1%
PRL	100%	0	0
PTZ	100%	0	0
SXT	94.5%	0	6.5%
IMP	100%	0	0
MEM	100%	0	0
Co	0	0	100%

*R: Resistant; **I:Intermediate; ***S: Sensitive

Table 3.3 Sampling Distribution of Isolates from Clinical Units

Clinical Servise	**Number (%)**
Burn Unit	28 (45.9%)
Anesthesia	27 (44.2%)
Brain-Nerve Surgery	4 (6.6%)
General Surgery	2 (3.3%)

The most prevalent clinical sample was blood from which *A.baumannii* isolates were recovered (Table 3.4). It is followed by wound (18.1%), burn wound (13.1%), and TTA (13.1%).

MIC values of meropenem and imipenem determined by E-test were given in table 3.5. CLSI gives the MIC interprevative standard (μg/mL) for both imipenem and meropenem; ≤ 4 as sensitive (S), 8 as intermediate (I), and ≥16 as resistant (R). All tested isolates were resistant to IMP and MEM. For the 61 isolates, the MIC_{50} of IMP and MEM were 16 and >32; the MIC_{90} were 192 and >32 respectively.

Table 3.4 Distribution of Isolates in Clinical Samples

Clinical Sample	Number (%)
Blood	25 (41.0%)
Wound	11 (18.1%)
Burn Wound	8 (13.1%)
Transtracheal Aspiration	8 (13.1%)
Catheter	3 (4.9%)
Urine	3 (4.9%)
Nasal sample	2 (3.3%)
Sputum	1 (1.6%)

Table 3.5 MIC (μg/mL) values of imipenem and meropenem by E-Test

No	IMP	MEM	No	IMP	MEM
1	16	>32	31	24	>32
2	16	>32	32	16	>32
3	16	>32	33	16	>32
4	16	>32	34	16	>32
5	16	>32	35	128	>32
6	16	>32	36	256	32
7	16	>32	37	24	>32
8	24	>32	38	>256	>32
9	16	>32	39	32	>32
10	24	>32	40	16	>32
11	32	>32	41	16	>32
12	48	>32	42	128	>32
13	32	>32	43	16	>32
14	128	>32	44	16	>32
15	32	>32	45	32	>32
16	16	>32	46	24	>32
17	256	>32	47	16	32
18	16	>32	48	16	>32
19	16	>32	49	32	32
20	16	>32	50	16	32

Table 3.5. Cont.

No	IMP	MEM		No	IMP	MEM
21	16	>32		**51**	48	>32
22	16	>32		**52**	256	>32
23	16	>32		**53**	48	32
24	16	>32		**54**	16	>32
25	16	>32		**55**	24	>32
26	16	32		**56**	256	>32
27	32	32		**57**	16	>32
28	16	>32		**58**	32	32
29	16	>32		**59**	256	>32
30	32	>32		**60**	64	>32
				61	192	>32

Using DDST, CDT, and MBL Etest, MBL activity was not found in any tested isolate. AmpC type enzyme was expressed by 13.1 % (n=8) of the carbapenem resistant *A. baumannii* strains. PCR amplifications indicated that among AmpC β-lactamase producing strains, 7 strains had OXA-51 and OXA-23; 1 strain had OXA-51 and OXA-58.

The positive rates of phenotypic tests to screen the carbapenemase and metallo-β-lactamase were given in Table3.6.

Table 3.6 Number of Positive Results from Phenotypic Tests

Test	**MHT**	**DDST**	**CDT**	**MBL Etest**	**AmpC (BA)**
Number (%)	61 (100%)	0 (0%)	0 (0%)	0 (0%)	8 (13.1%)

Using multiplex PCR assay, all strains were detected as having $bla_{OXA\text{-}51}$-like gene (figure 3.1 and table 3.7). Comparison of PCR results with MIC values were given in Appendix.

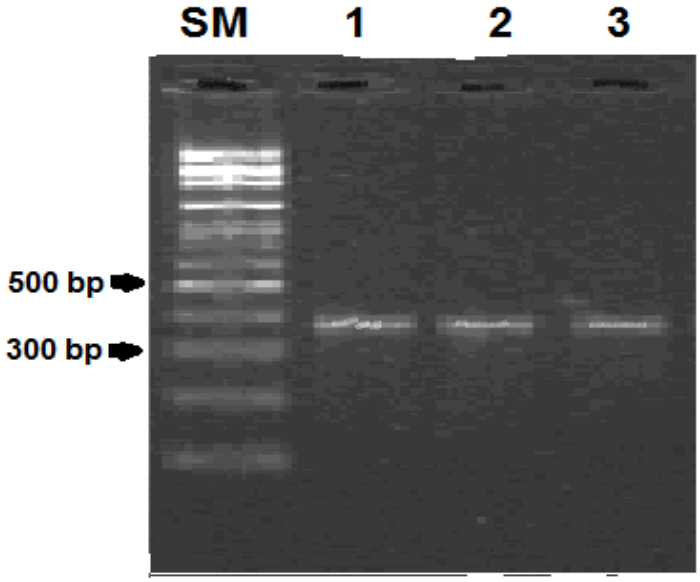

Figure 3.1 Examples of agarose gel. The numbers were given above indicate the *bla*$_{OXA51}$ positive samples. Lane SM, molecular size marker (MBI fermentas, 100bp DNA Ladder yielding the fragments from 100 to 1000bp), and sizes of 300 and 500 bp were indicated on the left.

According to sequence analysis of OXA-51, there were three different sequence types of OXA-51. In first group, 43.75 % of the strains have the sequence identical with OXA-64 (genebank accession number AY750907). Second group was OXA-66 (37.5%) having identical sequence with genebank accession number AY750908 described before by Brown and Amyes (2005). The last group was OXA-91 (18.75%) which was described as a new OXA-51-type enzyme by Koh *et al.* (2007) with genebank accesion number DQ519086. The obtained sequence of the strains having *bla*$_{OXA-51}$ genes and their comparison with the sequence from genebank were given in table 3.8.

Table 3.7 PCR test results

No.	OXA-51	OXA-23	OXA-58	OXA-24	No.	OXA-51	OXA-23	OXA-58	OXA-24
1	+	+	-	-	**31**	+	+	-	-
2	+	+	-	-	**32**	+	+	-	-
3	+	+	-	-	**33**	+	+	-	-
4	+	+	-	-	**34**	+	+	-	-
5	+	+	-	-	**35**	+	-	+	-

Table 3.7 Cont.

No.	OXA-51	OXA-23	OXA-58	OXA-24	No.	OXA-51	OXA-23	OXA-58	OXA-24
6	+	-	+	-	**36**	+	-	+	-
7	+	+	-	-	**37**	+	+	-	-
8	+	+	-	-	**38**	+	+	-	-
9	+	-	+	-	**39**	+	-	+	-
10	+	+	-	-	**40**	+	-	-	-
11	+	+	-	-	**41**	+	-	+	-
12	+	+	-	-	**42**	+	-	-	-
13	+	+	+	-	**43**	+	+	-	-
14	+	+	-	-	**44**	+	+	-	-
15	+	+	-	-	**45**	+	+	-	-
16	+	+	-	-	**46**	+	+	-	-
17	+	+	+	-	**47**	+	+	-	-
18	+	+	-	-	**48**	+	+	-	-
19	+	+	-	-	**49**	+	+	-	-
20	+	+	-	-	**50**	+	+	-	-
21	+	+	-	-	**51**	+	+	-	-
22	+	+	-	-	**52**	+	+	-	-
23	+	+	-	-	**53**	+	+	-	-
24	+	+	-	-	**54**	+	+	-	-
25	+	+	-	-	**55**	+	+	-	-
26	+	+	-	-	**56**	+	+	+	-
27	+	-	+	-	**57**	+	+	-	-
28	+	-	+	-	**58**	+	+	-	-
29	+	+	-	-	**59**	+	+	-	-
30	+	-	-	-	**60**	+	+	-	-
					61	+	+	-	-

Table 3.8.a. Nucleotide sequences of samples having OXA-64

```
#AY750907      TTG AGC ACC ATA AGG CAA CCA CCA CAG AAG TAT TTA AGT GGG ACG GGC AAA AAA GGC TAT TCC CAG AAT GGG AAA AGG
#AJ309734      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ...
#S-OXA51        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ...

#AY750907      ACA TGA CCC TAG GCG ATG CTA TGA AAG CTT CCG CTA TTC CGG TTT ATC AAG ATT TAG CTC GTC GTA TTG GAC TTG AAC
#AJ309734      ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.
#S-OXA51       ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.

#AY750907     GTC CTT TAA AAA TTA CTC CTC AGC AAG AGG CAC AGT TTG CTT ACA AGC TAG CTA ATA AAA CGC TTC CAT TTA GCC AAA
#AJ309734     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#S-OXA51      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#AY750907      GTC CTT TAA AAA TTA CTC CTC AGC AAG AGG CAC AGT TTG CTT ACA AGC TAG CTA ATA AAA CGC TTC CAT TTA GCC CAA
#AY750907      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..
#AJ309734      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..
#S-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..

#AY750907      AAG TCC AAG
#AJ309734      ... ... ...
#S-OXA51       ... ... ...
```

Table 3.8.b Nucleotide sequences of OXA-91

```
#DQ392963 TTG AGC ACC ATA AGG CAA CCA CCA CAG AAG TAT TTA AGT GGG ATG GTA AAA AAA GGT TAT TCC CAG AAT GGG AAA AGG
#DQ519086 ... ... ... ... ... ... ... .T. ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ..A
#1-OXA51  ... ... ... ... ... ... ... .T. ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ..A


#DQ392963 ACA TGA CCC TAG GCG ATG CCA TGA AAG CTT CCG CTA TTC CGG TTT ATC AAG ATT TAG CTC GTC GTA TTG GAC TTG AGC
#DQ519086 ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.
#1-OXA51  ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.

#DQ392963  TCA TGT CTA AGG AAG TGA AGC GTG TTG GTT ATG GCA ATG CAG ATA TCG GTA CCC AAG TCG ATA ATT TTT GGC TGG TGG
#DQ519086  ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#1-OXA51   ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ...

#DQ392963  GTC CTT TAA AAA TTA CTC CTC AGC AAG AGG CAC AGT TTG CTT ACA AGC TAG CTA ATA AAA CGC TTC CAT TTA GCC AAA
#DQ519086      ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#1-OXA51       ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ..G

#DQ392963   AAG TCC AAG
#DQ519086   ... ... ...
#1-OXA51   ... ... ...
```

Table 3.8.c Nucleotide sequences of OXA-66

```
DQ392963  TTG AGC ACC ATA AGG CAA CCA CCA CAG AAG TAT TTA AGT GGG ATG GTA AAA AAA GGT TAT TCC CAG AAT GGG AAA AGG
#AY750909 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#6-OXA51  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...


#DQ392963 ACA TGA CCC TAG GCG ATG CCA TGA AAG CTT CCG CTA TTC CGG TTT ATC AAG ATT TAG CTC GTC GTA TTG GAC TTG AGC
#AY750909 ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ...
#6-OXA51  ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ...


#DQ392963 TCA TGT CTA AGG AAG TGA AGC GTG TTG GTT ATG GCA ATG CAG ATA TCG GTA CCC AAG TCG ATA ATT TTT GGC TGG TGG
#AY750909 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#6-OXA51  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#DQ392963 GTC CTT TAA AAA TTA CTC CTC AGC AAG AGG CAC AGT TTG CTT ACA AGC TAG CTA ATA AAA CGC TTC CAT TTA GCC AAA
#AY750909 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#6-OXA51  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#DQ392963 AAG TCC AAG
#AY750909 ... ... ...
#6-OXA51  ... ... ...
```

Table 3.9. Sequence of sample having OXA-23 and comparison with AJ132105

```
# AY795964  ATG AAT AAA TAT TTT ACT TGC TAT GTG GTT GCT TCT CTT TTT CTT TCT GGT TGT ACG GTT CAG CAT AAT TTA ATA AAT
# AJ132105  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

# AY795964   GAA ACC CCG AGT CAG ATT GTT CAA GGA CAT AAT CAG GTG ATT CAT CAA TAC TTT GAT GAA AAA AAC ACC TCA GGT GTG
# AJ132105   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

# AY795964  CTG GTT ATT CAA ACA GAT AAA AAA ATT AAT CTA TAT GGT AAT GCT CTA AGC CGC GCA AAT ACA GAA TAT GTG CCA GCC
# AJ132105  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

# AY795964  TCT ACA TTT AAA ATG TTG AAT GCC CTG ATC GGA TTG GAG AAC CAG AAA ACG GAT ATT AAT GAA ATA TTT AAA TGG AAG
# AJ132105  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23   --- --- --- --- --- --- --- --- --- --- --- --- --- --. ... ... ... ... ... ... ... ... ... ... ... ...

# AY795964  GGC GAG AAA AGG TCA TTT ACC GCT TGG GAA AAA GAC ATG ACA CTA GGA GAA GCC ATG AAG CTT TCT GCA GTC CCA GTC
# AJ132105  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#AY795964   TAT CAG GAA CTT GCG CGA CGT ATC GGT CTT GAT CTC ATG CAA AAA GAA GTA AAA CGT ATT GGT TTC GGT AAT GCT GAA
#AJ132105   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

# AY795964  ATT GGA CAG CAG GTT GAT AAT TTC TGG TTG GTA GGA CCA TTA AAG GTT ACG CCT ATT CAA GAG GTA GAG TTT GTT TCC
# AJ132105  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#AY795964  CAA TTA GCA CAT ACA CAG CTT CCA TTT AGT GAA AAA GTG CAG GCT AAT GTA AAA AAT ATG CTT CTT TTA GAA GAG AGT
#AJ132105  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

# AY795964 AAT GGC TAC AAA ATT TTT GGA AAG ACT GGT TGG GCA ATG GAT ATA AAA CCA CAA GTG GGC TGG TTG ACC GGC TGG GTT

# AJ132105 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#27-OXA23  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

# AY795964 GAG CAG CCA GAT GGA AAA ATT GTC GCT TTT GCA TTA AAT ATG GAA ATG CGG TCA GAA ATG CCG GCA TCT ATA CGT AAT

# AJ132105 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#27-OXA23  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..- --- --- --- --- --- --- --- --- --- ---
```

Table 3.9 Cont.

```
# AY795964 GAA TTA TTG ATG AAA TCA TTA AAA CAG CTG AAT ATT ATT TAA
# AJ132105 ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#27-OXA23  --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

Table 3.10. Sequence of Samples having OXA-58 and comparison with HQ219687

```
#HQ219687 TGA GCA TAG TAT GAG TCG AGC AAA AAC AAG TAC AAT TCC ACA AGT GAA TAA CTC AAT CAT CGA TCA GAA TGT TCA AGC
#33-OXA58 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#HQ219687 GCT TTT TAA TGA AAT CTC AGC TGA TGC TGT GTT TGT CAC ATA TGA TGG TCA AAA TAT TAA AAA ATA TGG CAC GCA TTT
#33-OXA58 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#HQ219687 AGA CCG AGC AAA AAC AGC TTA TAT TCC TGC ATC TAC ATT TAA AAT TGC CAA TGC ACT AAT TGG TTT AGA AAA TCA TAA
#33-OXA58 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#HQ219687 AGC AAC ATC TAC AGA AAT ATT TAA GTG GGA TGG AAA GCC ACG TTT TTT TAA AGC ATG GGA CAA AGA TTT TAC TTT GGG
#33-OXA58 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#HQ219687 CGA AGC CAT GCA AGC ATC TAC AGT GCC TGT ATA TCA AGA ATT GGC ACG TCG TAT TGG TCC AAG CTT AAT GCA AAG TGA
#33-OXA58 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#HQ219687 ATT GCA ACG TAT TGG TTA TGG CAA TAT GCA AAT AGG CAC GGA AGT TGA TCA ATT TTG GTT GAA AGG GCC TTT GAC AAT
33-OXA58  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#HQ219687 TAC ACC TAT ACA AGA AGT AAA GTT TGT GTA TGA TTT AGC CCA AGG GCA ATT GCC TTT TAA ACC TGA AGT TCA GCA ACA
#HQ005471 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
33-OXA58  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#HQ219687 AGT GAA AGA GAT GTT GTA
#33-OXA58 ... ...
```

Most of the *A .baumannii* strains (n=55) (90.1%) had at least two types of carbapenemase. One of them was intrinsic and the other was acquired. Five percent (n=3) of the isolates had only intrinsic OXA-51 enzyme. Three isolates (4.9%) had three genes which encode OXA-51, OXA-23, and OXA-58.

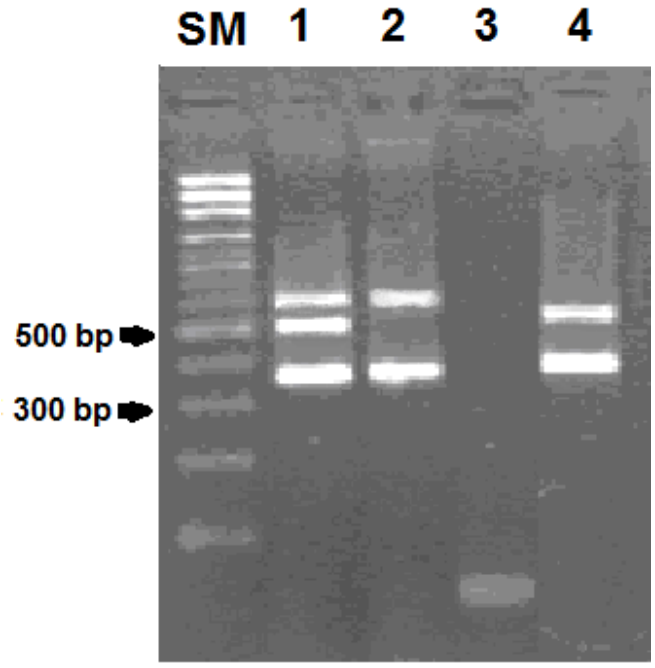

Figure 3.1 Examples of agarose gels.. Lane SM, molecular size marker (MBI fermentas, 100bp DNA Ladder yielding the fragments between 100-1000bp), and the sizes of 300 and 500 bp were indicated on the left. Lane 1 is the sample having bla_{OXA51}, bla_{OXA23}, and bla_{OXA58}. lane 2 is the sample having bla_{OXA51} and bla_{OXA58}, lane 4 is the sample having bla_{OXA51} and bla_{OXA23}. Lane 3 is negative control.

Among acquired carbapenemases, we found that most carbapenem resistant *A.baumannii* strains (81.9%) from ICUs of Gulhane Military Medical Academy had bla_{OXA-23} gene (n=50). bla_{OXA-23} sequence was 100% identical with the ARI-1 original sequence of genebank accesion number AJ132105 defined by Donald *et al.* in 2000. Sequence of samples having OXA-23 was given in table 3.9.

Eleven *A.baumannii* strains carried carbapenem-hydrolyzing oxacillinase OXA-58 (bla_{OXA-58}) gene (n=11). Sequence obtained from OXA-58 was identical

with HQ219687 Genbank accession number. Sequence of samples having OXA-58 was given in table 3.10.

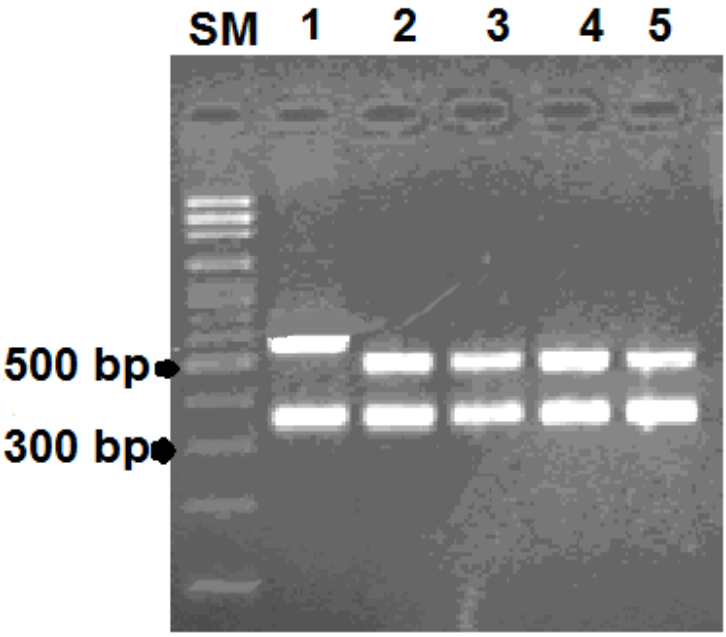

Figure 3.1 Examples of agarose gel. Lane SM, molecular size marker (MBI fermentas, 100bp DNA Ladder yielding the fragments between 100-1000bp), and the sizes of 300 and 500 bp were indicated on the left. Lane 1 is the sample having *bla*$_{OXA51}$ and *bla*$_{OXA58}$. Lane 2,3,4,5 are the samples having *bla*$_{OXA51}$ and *bla*$_{OXA23}$.

Enzymes inferred from PCR analysis were given in table 3.11. The most prevalent enzymes infered from PCR assay were OXA-51+OXA-23 (77.0%). OXA-24 enzymes were not found in any strains included in the study.

Table 3.11. Enzymes inferred from PCR

Enzyme	**N (%)**
OXA-51-like only	3 (4.9%)
OXA-23 with OXA-51	47 (77.0%)
OXA-58 with OXA-51	8 (13.1%)
OXA-23 and OXA-58 with OXA-51	3 (4.9%)
OXA-24-like	0

CHAPTER IV

DISCUSSION

A.baumannii has become a very important hospital-acquired pathogen. They cause outbreaks particularly in intensive care units (ICUs). They may cause various infections; such as, pneumonia, UTIs and septicemia (Bergogne-Berezin and Towner, 1996). Strains of *A.baumannii* have been detected to be resistant to most classes of clinically relevant antibiotics. Aminoglycosides, $3^{Th.}$ generation cephalosporins, and fluoroquinolones are some examples of them. Treatments of such infections are very difficult to succed because of the multi-drug resistance (Bergogne-Berezin and Towner, 1996; Poirel and Nordmann, 2006). Imipenem and meropenem are generally preferred as the last resort in treatment of these infections. On the other hand, carbapenem resistance in *A.baumannii* has been reported increasingly (Poirel and Nordmann, 2006). This problem is becoming more prevalent and resistant strains should be typed strict precausions should be taken to avoid the problem.

Carbapenem resistant isolates are generally resistant to most other classes of antibiotics, while usually retaining susceptible to tigecycline and colistin (Queenan and Bush, 2007). Colistin resistance was not observed in any 61 tested isolates. In this study tygecyclin was not tested. All isolates have high resistance rates against other antimirobial agents including amikacin (AN), ampicillin/sulbactam (SAM), cefepime (FEB), cefotaxime (CTX), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GN), piperacillin (PRL), piperacillin/tazobactam (PTZ), and trimethoprim/ sulfamethoxazole (SXT). The resistance rates were 90.2%, 65.7%, 92%, 90.2%, 88.5%, 94.1%, 83.7%, 100%, 100%, and 94.5% respectively.

A study from Spain also indicated that the imipenem resistant isolates have higher resistance rates against other agents than imipenem susceptible strains (Oteo *et al.,* 2007). They also indicated the high level of sulbactam resistance with 14%. Sulbactam is a good choise in treatment infections *A.baumannii* because of its high intrinsic activity. It has bactericidal effect on bacterial cells. In our study carbapenem resistance with sulbactam resistance was a noteworthy problem. Imipenem resistant strains were susceptible by only 11.4% to SAM only. Our 24 (39.3 %) strains were resistant to all tested antimicrobial agents except colistin.

Resistance against carbapenem in and on itself is considered sufficient to define an *A.baumannii* isolate as highly resistant (Turton *et al.,* 2007). This study confirmed the idea. Strains demonstrating resistance to all commercially available antimicrobial agents have also been reported. This high level of resistance makes treatment difficult sometimes impossible.

One of the resistance mechanism including enzymatic activation, active efflux of agent or modification of binding proteins may induce carbapenem resistance in *A. baumannii* (Queenan and Bush, 2007). The most active mechanism that induces IMP and MEM resistance in *A. baumannii* strains is expression β-lactamases which hydrolyse carbapenem. Different types of carbapenemases have been identified in *A. baumannii*. Class B metallo-β-lactamases and class D OXA-type β-lactamases are the most prevalent types. Regulation of AdeABC efflux system, PBP or porin modification which cause reduction of susceptibility to related drugs are less common in acinetobacers (Queenan and Bush, 2007).

Carbapenem resistance because of the OXA-type (class D) carbapenemases is growing problem (Gur *et.al.*, 2008; Carvalho *at.al.,* 2009; Kuo *et.al.,* 2010) and OXA enzymes are the most important reason for resistance to IMP and MEM in acinetobacters worldwide (Poirel and Nordmann, 2006).

Using multiplex PCR assay, we detected all strains included in this study had $bla_{OXA\text{-}51}$-like genes. According to sequence analysis, there were three different sequence types of OXA-51. In first group, 43.75 % of the strains have the sequence identical with AJ309734 genebank accesion number, with the previously reported by Brown *et al.* from Arjentina. These strains were OXA-64 (genebank accession

number AY750907). Second group of OXA-51 were OXA-66 (37.5%) having identity sequence with genebank accession number AY750908 described by Brown and Amyes (2005). The last group was OXA-91 (18.75) which was described as a new OXA-51-type enzyme by Koh *et al.* with genebank accesion number DQ519086 (2007). Since OXA-51 is ubiquitous in *A .baumannii,* there was no correlation between the presence of intrinsic $bla_{\text{OXA-51}}$ gene and the MIC values of carbapenems obtained from isolates included this study. On the other hand, the presence of OXA-23, OXA-24 and OXA-58 type enzymes indicated high level of carbapenem resistance (Woodford *et.al,* 2006). Our study showed that, 55 of 61 (90%) *A .baumannii* isolates had two, 3 (5%) isolates had three genes encoding oxa-carbapenemase, one intrinsic and the others acquired. Five percent (n=3) of the isolates had only intrinsic OXA-51 enzyme.

It has been known that $bla_{\text{OXA-51}}$ gene encoding cromosomal carbapenemase are carried by most *A. baumannii* strains. OXA-51 enzymes were firs accepted as not a major contributory factor for resistance to β-lactam antibiotics (Herritier *et al*., 2005). Turton (2010) have found that isolates with an insertion sequence, IS*AbaI* on $bla_{\text{OXA-51}}$ gene showed high level of resistance to carbapenems. IS*AbaI* insertion sequence provides a promoter for hyperproduction of carbapenemases (Brown and Amyes, 2006). *A.baumannii* strains have some other OXA carbapenemase genes belonging to $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, and $bla_{\text{OXA-58}}$ families. They are acquired by plasmids or other mobile genetic elements. MBLs are generally accepted as more efficient hydrolyser of IMP and MEM in vitro, but in acinetobacters, OXA-type carbapenemases can be more effective against IMP and MEM; and IS*Aba* sequence can induce high level of resistance in this strains (Brown and Amyes, 2006; Woodford *et al.,* 2006).

Five percent (n=3) of the isolates had only intrinsic OXA-51 enzyme. The 3 isolates having only OXA-51 should be investigated for other carbapenem resistance mechanism including efflux pump. Huang and co-workers (2008) found that MEM resistance may be mediated by over expression of AdeABC efflux pump system. They studied with isolates from surgical ICUs. AdeABC efflux expression is

conducted by AdeRS. Any mutations in AdeR or S may induce the expression of efflux proteins (Huang *et al.,* 2008).

In *Acinetobacter* strains, OXA-23 has been detected in isolates from many countries including Brazil "(Dalla-Costa *et al.,* 2003)", Singapore "(Koh *et al.* 2007)", Colombia "(Villegas *et al.,* 2007)", England "(Coelho *et al,* 2006)". According to the reports, OXA-23 oxacillinases was mostly prevalent in South America and Europe (Zarilli *et al.,* 2009). However, presence of OXA-23 was not sufficient to make these strains carbapenem resistant. Moreover, these OXA enzymes have not broad spectrum feature and they are not strong carbapenems hydrolyser as MBLs. MIC values of our strain varied between 16 to 256 mg/L. This may indicate other resistance mechanisms which restrict permeability of drugs may be the contributory factor for resistance (Brown and Amyes, 2006). We found that $bla_{\text{OXA-23}}$ gene was the major factor (81.9%) for IMP and MEM resistance in the strains from our hospital (n=50). The sequence of OXA-23 was 100% identical with the ARI-1 original sequence of genebank accesion number AJ132105 (Donald *et al.*, 1999).

Eleven *A.baumannii* strains carried carbapenem-hydrolyzing oxacillinase OXA-58 ($bla_{\text{OXA-58}}$) gene (n=11). Sequence obtained from OXA-58 was identical with accession number of Genbank HQ219687. The $bla_{\text{OXA-58}}$ was frequently found in Europe (Zarilli *et al.,* 2009). A study from Turkey showed that OXA-58 was found in 17 of the 18 isolates in Ankara, but only 1 of 26 isolates in İstanbul (Gur *et al.,* 2008). We, on the other hand, found 18% of *A.baumannii* strains carried carbapenem-hydrolyzing oxacillinase $bla_{\text{OXA-58}}$ gene (n=11) which is less than $bla_{\text{OXA-23}}$. Three of these eleven isolates having $bla_{\text{OXA-58}}$ gene also carried $bla_{\text{OXA-23}}$.Apparently the ratios of the resistance genes are changing depending on the source hospitals.

OXA-24 was detected strains from Spain. These Spain isolates had high level resistance to carbapenems (Brown and Amyes, 2006). OXA-25, -26 and -40 belonging to OXA-24 family; were detected in isolates from Belgium Portugal as weel as Spain (Brown and Amyes, 2006). We have not found any OXA-24 enzyme in our strains. Park *et al.* (2010) also did not detect OXA-24 and OXA-58 enzymes studying with 30 extensively drug-resistant *A .baumannii* isolates. They found that 77% of their isolates carried $bla_{\text{OXA-23}}$ gene.

Restricted permeability of membrane and expression of AmpC enzymes are other significant mechanism which cause IMP and MEM resistance in *A.baumannii* (Quale *et al.,* 2003). Damage of relevant OMP cause reduction in outer-membrane transport, but was not tested in our strains. In our study, 13.1 % of the carbapenem resistant *A. baumannii* strains were detected as AmpC producer. Therefore, the AmpC enzymes might be a contributing factor for carbapenem resistance among these isolates.

In various studies, several types of MBLs were detected in *A. baumannii*. MBL variants may have contribution in high level of carbapenem resistance in acinetobacters. E-test method is easy way to detect the presence of MBL activity in test strains (Poirel and Nordmann, 2006).

In the present study, by using DDS test and and MBL E-test we did not detect any MBL production although all strains have carbapenemase activity as it was shown through MHT. Production of MBL was not seemed as an important factor in carbapenem resistance among *A. baumannii* strains isolated from ICU of Gulhane Military Medical Academy Hospital. Similar to our case, Sinha and Srinivasa (2007) did not find MBL activity at their study investigating the issue on 150 clinical isolates of *Acinetobacter* species. They used double-disk approximation test to screen the MBL production. Villegas *et al*. (2007) also, investigated carbapenemases in *A .baumannii* strains from multy center study including ten hospitals and they did not find MBL activity. They used MBL E-test method to screen related enzymes. Another study from South Korea did not find MBL in 30 extented-drug resistant *A.baumannii* (Park *et al,* 2010).

4.1. Conclusion

- Resistance against carbapenem is seemed to be sufficient to define an *A.baumannii* isolate as highly resistant.
- Therapy of these infections is often difficult. Colistin may be effective in treatment of multiresistant infections

- Our data showed that production of carbapenem hydrolyzing oxacillinases by *A. baumannii* seems to constitute the main resistance problem in our source hospital.
- AmpC β-lactamase is also a contributory factor for carbapenem resistance among the isolates in this hospital.
- MBL production was not a factor in carbapenem resistance among *A. baumannii* strains included this study.
- *A. baumannii* infections in ICUs urged to need for better control. It is clear that there is a special necessity to search for potent inhibitor of Class D OXA-enzymes to prevent failure in treatment.

REFERENCES

Ambler RP. 1980 “The structure of beta-lactamases. Philos. Trans R. Soc. Lond. B 289:321-331.

Bergogne-Berezin E, and Towner KJ. 1996 “*Acinetobacter* species as nosocomial pathogens: microbiological, clinical and epidemiological features” Clin Microbiol Rev. 150:148-165.

Bergogne-Berezin E 2001 “The increasing role of *Acinetobacter* species as nosocomial pathogens” Curr. Infect. Dis Rep. 3, 440–444.

Bou G, Oliver A, Martinez-beltran J. 2000 “A novel class D β-lactamase (OXA-24) with carbapenemase activity in *Acinetobacter baumannii* clinical strains” Antimicrob Agents Chemother 44:1556-1561.

Brown S, Young HK, Amyes SG. 2005 “Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of Acinetobacter baumannii from Argentina” Clin Microbiol Infect. Jan;11(1):15-23.

Brown S and Amyes S 2006 “OXA β-lactamase in Acinetobacter: the story so far” 2006 Journal of Antimicrobial Chemotherapy vol.57, 1-3.

Carvalho KR**,** Carvalho-Assef AP**,** Peirano G**,** Santos LC**,** Pereira MJ**,** Asensi MD**.** 2009 “Dissemination of multidrug-resistant Acinetobacter baumannii genotypes carrying bla(OXA-23) collected from hospitals in Rio de Janeiro, Brazil” Int J Antimicrob Agents. 2009 Jul;34(1):25-8.

Clinical Laboratory Standards Institute. *Performance standards for antimicrobialsusceptibility testing* 18th. İnformation supplement (M100-S18) 2008.

Clinical Laboratory Standards Institute. *Performance standards for antimicrobialsusceptibility testing* 18th. İnformation supplement (M100-S20) 2010.

Coelho JM, Turton JF, Kaufmann ME, Glover J, Woodford N, Warner M, Palepou MF, Pike R, Pitt TL, Patel BC, Livermore DM. 2006 "Occurrence of carbapenem-resistant Acinetobacter baumannii clones at multiple hospitals in London and Southeast England" J Clin Microbiol. Oct;44(10):3623-7.

Coudron Philip E. 2005 "Inhibitor-Based Methods for Detection of Plasmid-Mediated AmpC β-Lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis" J Clin Microbiol.* ; 43(8)**:** 4163–4167.

Dalla-Costa LM, Coelho JM, Souza HA *et al.,* 2003 "Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the OXA-23 enzyme in Curitiba, Brazil" J Clin Microbiol 41: 3403-6.

Donald HM, Scaife W, Amyes SGB, Young HK 1999 "Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92" Antimicrobial Agents and Chemotherapy Jan 196-199.

Falagas Matthew E, Koletsi Patra K. and Bliziotis Ioannis A. 2006 "The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii and Pseudomonas aeruginosa"* J Med Microbiol 55 (2006), 1619-1629.

Fernandez-Cuenca F, martinez ML, Conejo MC *et al.,* 2003 "Relationship between β-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii"* J Antimicrob Chemother 51:565-74.

Ferreira ACB, Gobara S, Costa SF *et al.* 2004 “Emergence of resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* species after the use of antimicrobials for burned patients” Infection Control and Hospital Epidemiology Vol.25(10):868-872.

Forbes Betty Ann, Sahm Daniel F., & Alice Weissfeld 2002 “Bailey & Scott's Diagnostic Microbiology, 11th Edition”.

Gales AC, Reis AO, Jones RN. 2001 “Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines” *J Clin Microbiol*;39:183-90.

Gillespie Stephen H. and Hawkey Peter M. 2006 “Principles and Practice of Clinical Bacteriology Second Edition” John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, England.

Gur Deniz, Korten Volken, Unal Serhat, Lalitagauri M. Deshpande, and Mariana Castanheira 2008 “Increasing carbapenem resistance due to the clonal dissemination of oxacillinase (OXA-23 and OXA-58)-producing *Acinetobacter baumannii*: report from the Turkish SENTRY Program sites” J. Med. Microbiol., 57: 1529 - 1532.

Herritier C, Poirel L, Fournier PE *et al.* 2005 “Characterization of the naturally occuring oxacillinases of *Acinetobacter baumannii*” Antimicrob Agents Chemother 49:4174-9.

Henwood, Caroline J, Tess Gatward., Marina Warner Mark W. Stockdale, Richard P. Spence, Kevin J. Towner, David M. Livermore, and Neil Woodford 2002 “Antibiotic resistance among clinical isolates of *Acinetobacter* in the UK, and *in vitro* evaluation of tigecycline” J. Antimicrob. Chemother. 49(3): 479-487.

Huang L, sun L, Xu G, Xia T 2008 “Differential susceptibility to carbapenem due to the AdeABC efflux pump among nosocomial outbreak isolates of *Acinetobacter baumannii* in a Chinese hospital” Diagn Microbiol Infect Dis. 62(3):326-32.

Karageorgopoulos DE. and Falagas ME. 2008 “Current control and treatment of multidrug-resistant *Acinetobacter baumannii* infections” Lancet Infect. Dis. Vol.8:751-62.

Koch Arthur L. 2006 “The Bacteria: Their Origin, Structure, Function and Antibiosis” *by Indiana University, Bloomington, IN, USA.*

Koh TH**,** Sng LH**,** Wang GC**,** Hsu LY**,** Zhao Y**.** 2007 “IMP-4 and OXA beta-lactamases in *Acinetobacter baumannii* from Singapore” J Antimicrob Chemother. Apr;59(4):627-32.

Koneman E, Winn W, Allen S, Janda W, Procop G *et al.,* 2006 “Koneman’s Color Atlas and Textbook of Diagnostic Microbiology” Sixth edition. Lippincott Williams & Wilkins, USA.

Kuo HY, Yang CM, Lin MF, Cheng WL, Tien N, Liou ML. 2010 “Distribution of blaOXA-carrying imipenem-resistant Acinetobacter spp. in 3 hospitals in Taiwan” Diagn Microbiol Infect Dis. 2010 Feb;66(2):195-9.

Lee K, Lim YS, Yong D, Yum JH, Chong Y. 2003 “Evaluation of the Hodge test and the imipenem-EDTA double disk synergy test for differentiation of metallo-β-lactamases producing clinical isolates of *Pseudomonas* spp and *Acinetobacter* spp.” J Clin Microbiol 2003;41:4623-9.

Limansky AS, Mussi MA, Viale AM, 2002 “Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance” J Clin Microbiol 40:4776-8.

Livermore D.M., Woodford N. 2000 “Carbapenemases: A problem in waiting?” Current Opinion in Microbiology Vol. 3 (5), 489-495.

Liu S.Y., J.Y. Lin, C. Chu, L.H. Su, T.Y. Lin, C.H. Chiu 2006 “Integron-associated imipenem resistance in *Acinetobacter baumannii* isolated from a regional hospital in Taiwan” International Journal of Antimicrobial Agents, Vol. 27, Issue 1, 81-84.

Murrey PR, Rosenthal KS, Pfaller MA 2010 “Tıbbi Mikrobiyoloji 6. baskı, Türkçe Çevirisi” Atlas Kitapçılık, Ankara.

Noyal MJC, Menezes GA, Harish BN, Sujatha S, Parija SC. 2009 “Simple screening tests for detection of carbapenemases in clinical isolates of nonfermantative Gram-negative bacteria” Indian J Med Res 129:707-712.

Oteo J, Garcia-Estebanez C, Miguelanez S *et al.,* 2007 “Genotypic diversity of imipenem resistant isolates of *Acinetobacter baumannii* in Spain” Journal of Infection 55:260-266.

Park YS, Lee H, Lee KS *et.al.* 2010 “Extensively drug-resistant *Acinetobacter baumannii*: risk factors for acquisition and prevalent OXA-type carbapenemases-a multicentre study” Internal Journal of Antimicrobial Agents 36:430-435.

Poirel L, Nordmann P. Carbapenem resistance in Acinetobacter baumannii: mechanisms and epidemiology. 2006 Clin Microbiol Infect. Sep;12(9):826-36. Review.

Roberts Marilyn C 2006 “Multidrug-resistant genes are associated with an 86-kb island in *Acinetobacter baumannii”* Trends in Microbiology Vol.14 No.9:375-378.

Quale J, Bratu S, Landman D *et.al.,* 2003 “Molecular epidemiology and mechanism of carbapenem resistance in *Acinetobacter baumannii* endemic in New York city” Clin Infect Dis 37:214-20.

Queenan Anne Marie and Bush Karen 2007 “Carbapenemases: the Verstile β-Lactamases” Clinical microbiology reviews, Vol.20(3) 440-458.

Sinha M, Srinivasa H. 2007 “Mechanism of resistance to carbapenems in meropenem-resistant *Acinetobacter* isolates from clinical samples” Indian J Med Microbiol 25:121-5.

Turton JF, Gabriel SN, Valderrey C, Kaufmann ME, Pitt TL 2007 “Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*” Clin Microbiol ınfect 13:807-815.

Turton Jane F 2010 “The role of ISAba1 in expression of OXA carbapenemase genes in Acinetobacter baumannii” FEMS Microbiol Lett 258:72-7.

Villegas M.V., J.N. Kattan, A.C., Lolans K, Guzman A.M, Woodford N., Livermore D., John P. Quinn and the Colombian Nosocomial Bacterial Resistance Study Group 2007 “Dissemination of *Acinetobacter baumannii* Clones with OXA-23 Carbapenemase in Colombian Hospitals” Antimicrob. Agents Chemother. Vol.51: 2001 - 2004.

Walther-Rasmussen Jan and Niels Høiby 2006 “OXA-type carbapenemases” Journal of Antimicrobial Chemotherapy 57, 373–383.

Woodford Neil, Matthew J. Ellington, Juliana M. Coelho, Jane F. Turton, M. Elaina Ward, Susan Brown, Sebastian G.B. Amyes, David M. Livermore 2006 “Multiplex PCR for genes encoding prevalent OXA carbapenemases in Acinetobacter spp” International Journal of Antimicrobial Agents, Vol. 27, Issue 4, 351-353.

Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. 2002 “”Imipenem-EDTA disk method for differentiation of metallo-β-lactamases producing clinical isolates of *Pseudomonas* spp and *Acinetobacter* spp.” J Clin Microbiol vol.40:3798-801.

Zarrilli Raffaele **,** Maria Giannouli**,** Federica Tomasone**,** Maria Triassi and Athanassios Tsakris 2009 “Carbapenem resistance in Acinetobacter baumannii: the molecular epidemic features of an emerging problem in health care facilities” J Infect Developing Countries Vol.3(5):335-41**.**

Zarrilli R, Casillo R, Di Popolo A, Tripodi MF, Bagattini M, Cuccurullo S, Crivaro V, Ragone E, Mattei A, Galdieri N, Triassi M, Utili R. 2007 “Molecular epidemiology of a clonal outbreak of multidrug-resistant Acinetobacter baumannii in a university hospital in Italy” Clin Microbiol Infect. May;13(5):481-9.

APPENDIX A

AGE AND GENDER OF PATIENTS

Table A.1.Distribution of Age and Gender.

No.	Age	Gender
1	2	M
2	21	M
3	23	M
4	40	M
5	44	M
6	2	W
7	33	M
8	34	W
9	30	M
10	43	M
11	55	M
12	32	M
13	34	M
14	68	W
15	71	W
16	24	M
17	27	M
18	53	M
19	47	W
20	33	M

Table A.1. Cont.

21	22	M
22	24	M
23	24	M
24	30	W
25	20	M
26	26	M
27	24	M
28	79	M
29	71	M
30	21	M
31	22	M
32	30	M
33	27	W
34	30	M
35	32	W
36	36	W
37	66	M
38	33	W
39	44	W
40	21	M
41	21	M
42	22	W
43	83	M
44	33	M
45	68	M
46	45	M
47	24	M
48	25	M
49	65	M

Table A.1. Cont.

No.	**Age**	**Gender**
50	21	M
51	38	M
52	22	M
53	27	W
54	20	M
55	20	M
56	56	W
57	51	W
58	29	M
59	21	M
60	23	M
61	71	W

APPENDIX B

SAMPLES AND CLINICAL SERVICES

Table B.1. Sampling Distribution of Isolates from Clinical Services

No.	**Hospital service**	**Specimen Type**
1	Burn Unit	Burn Wound.
2	Burn Unit	Burn Wound
3	Burn Unit	Burn Wound
4	Burn Unit	Blood
5	Burn Unit	Burn Wound
6	Burn Unit	Burn Wound
7	Burn Unit	Wound
8	Anest	Blood
9	Anest	Wound
10	Anest	Wound
11	Anest	Blood
12	Burn Unit	Blood
13	Burn Unit	Blood
14	Anest	Blood
15	Anest	TTA
16	Burn Unit	Blood
17	Burn Unit	Blood
18	Anest	Blood
19	Burn Unit	Blood
20	Burn Unit	Blood
21	Burn Unit	Wound
22	Burn Unit	Urine
23	Burn Unit	Urine

Table B.1. Cont.

No.	Hospital service	Specimen Type
24	Brain-Nerve Surgery	Blood
25	General Surgery	Blood
26	Burn Unit	Blood
27	Burn Unit	Burn Wound
28	Anest	TTA
29	Anest	balgam
30	Brain-Nerve Surgery	Wound
31	Burn Unit	Wound
32	Anest	Wound
33	General Surgery	Burun
34	Anest	TTA
35	Burn Unit	katater
36	Anest	Burun
37	Anest	Blood
38	Anest	TTA
39	Burn Unit	Wound
40	Anest	Blood
41	Burn Unit	Blood
42	Anest	TTA
43	Anest	Blood
44	Anest	Wound
45	Anest	Blood
46	Burn Unit	Blood
47	Anest	TTA
48	Anest	Chatater
49	Brain-Nerve Surgery	Urine
50	Brain-Nerve Surgery	Wound

Table B.1. Cont.

No.	Hospital service	Specimen Type
51	Burn Unit	Blood
52	Burn Unit	Blood
53	Anest	TTA
54	Anest	Blood
55	Anest	Blood
56	Burn Unit	Wound
57	Burn Unit	Wound
58	Anest	Blood
59	Burn Unit	Wound
60	Anest	Chatater
61	Anest	TTA

APPENDIX C

MIC VALUES AND OXA-ENZYMES

Table C.1. Comparison of MIC values with enzymes infered from PCR assay

No.	**IMP**	**MEM**	**OXA-51**	**OXA-23**	**OXA-58**	**OXA-24**
1	16	>32	+	+	-	-
2	16	>32	+	+	-	-
3	16	>32	+	+	-	-
4	16	>32	+	+	-	-
5	16	>32	+	+	-	-
6	16	>32	+	-	+	-
7	16	>32	+	+	-	-
8	24	>32	+	+	-	-
9	16	>32	+	-	+	-
10	24	>32	+	+	-	-
11	32	>32	+	+	-	-
12	48	>32	+	+	-	-
13	32	>32	+	+	+	-
14	128	>32	+	+	-	-
15	32	>32	+	+	-	-
16	16	>32	+	+	-	-
17	256	>32	+	+	+	-
18	16	>32	+	+	-	-
19	16	>32	+	+	-	-
20	16	>32	+	+	-	-
21	16	>32	+	+	-	-
22	16	>32	+	+	-	-

Table C.1. Cont.

No.	IMP	MEM	OXA-51	OXA-23	OXA-58	OXA-24
23	16	>32	+	+	-	-
24	16	>32	+	+	-	-
25	16	>32	+	+	-	-
26	16	32	+	+	-	-
27	32	32	+	-	+	-
28	16	>32	+	-	+	-
29	16	>32	+	+	-	-
30	32	>32	+	-	-	-
31	24	>32	+	+	-	-
32	16	>32	+	+	-	-
33	16	>32	+	+	-	-
34	16	>32	+	+	-	-
35	128	>32	+	-	+	-
36	256	32	+	-	+	-
37	24	>32	+	+	-	-
38	>256	>32	+	+	-	-
39	32	>32	+	-	+	-
40	16	>32	+	-	-	-
41	16	>32	+	-	+	-
42	128	>32	+	-	-	-
43	16	>32	+	+	-	-
44	16	>32	+	+	-	-
45	32	>32	+	+	-	-
46	24	>32	+	+	-	-
47	16	32	+	+	-	-
48	16	>32	+	+	-	-
49	32	32	+	+	-	-

Table C.1. Cont.

No.	**IMP**	**MEM**	**OXA-51**	**OXA-23**	**OXA-58**	**OXA-24**
50	16	32	+	+	-	-
51	48	>32	+	+	-	-
52	256	>32	+	+	-	-
53	48	32	+	+	-	-
54	16	>32	+	+	-	-
55	24	>32	+	+	-	-
56	256	>32	+	+	+	-
57	16	>32	+	+	-	-
58	32	32	+	+	-	-
59	256	>32	+	+	-	-
60	64	>32	+	+	-	-
61	192	>32	+	+	-	-

APPENDIX D

PHENOTYPIC TESTS

Table D.1. Phetotypic Test Results of the Isolates

No.	MHT	DDST	CDT	MBL E-Test	BA Test
1	+	-	-	-	-
2	+	+	+	-	-
3	+	-	-	-	-
4	+	-	-	-	-
5	+	-	-	-	-
6	+	-	-	-	-
7	+	-	-	-	-
8	+	-	-	-	+
9	+	-	-	-	-
10	+	-	-	-	+
11	+	-	-	-	-
12	+	-	-	-	-
13	+	-	-	-	-
14	+	-	-	-	+
15	+	-	-	-	-
16	+	-	-	-	-
17	+	-	-	-	-
18	+	-	-	-	+
19	+	-	-	-	-
20	+	+	+	-	-
21	+	-	-	-	-
22	+	-	-	-	-

Table D.1. Cont.

No.	MHT	DDST	CDT	MBL E-Test	BA Test
23	+	-	-	-	-
24	+	-	-	-	-
25	+	-	-	-	-
26	+	-	-	-	-
27	+	-	-	-	-
28	+	+	-	-	+
29	+	-	-	-	-
30	+	-	-	-	-
31	+	-	-	-	-
32	+	+	+	-	-
33	+	-	-	-	-
34	+	-	-	-	+
35	+	+	+	-	-
36	+	-	-	-	-
37	+	-	-	-	-
38	+	-	-	-	-
39	+	-	-	-	-
40	+	-	-	-	-
41	+	-	-	-	-
42	+	-	-	-	-
43	+	-	-	-	-
44	+	-	-	-	-
45	+	-	-	-	-
46	+	-	-	-	-
47	+	-	-	-	+
48	+	-	-	-	-
49	+	-	-	-	-

Table D.1. Cont.

No.	MHT	DDST	CDT	MBL E-Test	BA Test
50	+	-	-	-	-
51	+	-	+	-	-
52	+	-	-	-	-
53	+	-	-	-	-
54	+	-	-	-	+
55	+	-	-	-	-
56	+	-	-	-	-
57	+	-	-	-	-
58	+	-	-	-	-
59	+	-	-	-	-
60	+	-	-	-	-
61	+	-	-	-	-

APPENDIX E

NUCLEOTIDE SEQUENCES OF OXA-51 GROUPS

Table E.1. Comparison of OXA 51 Sequences with reference accession numbers of GenBank

```
#DQ392963      TTG AGC ACC ATA AGG CAA CCA CCA CAG AAG TAT TTA AGT GGG ATG GTA AAA AAA GGT TAT TCC CAG AAT GGG AAA AGG
#AY750911      ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .GG ... ... ..C ... ... ... ... ... ... ..A
#AY750907      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ...
#AY750909      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519086      ... ... ... ... ... ... ... .T. ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ..A
#DQ519088      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519089      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
#DQ519090      .A. .AA .T. ... .A. ... .AT .T. ... ..A ... ... ... ... ... .A. .GC C.C .T. .T. .TA A.. C.. ... .C. .A.
#AJ309734      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ...
#1-OXA51       ... ... ... ... ... ... ... .T. ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ..A
#2-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. .GC ... ... ..C ... ... ... ... ... ... ...
#6-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#DQ392963      ACA TGA CCC TAG GCG ATG CCA TGA AAG CTT CCG CTA TTC CGG TTT ATC AAG ATT TAG CTC GTC GTA TTG GAC TTG AGC
#AY750911      ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AY750907      ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.
#AY750909      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ...
#DQ519086      ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.
#DQ519088      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519089      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ...
#DQ519090      .TT .T. .TT .G. ... .A. ... ..C ... .A. .TA .AG .G. .T. .A. ... ... .A. .G. .A. ... ... ... .T. CAA GCT
#AJ309734      ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.
#1-OXA51       ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.
#2-OXA51       ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.
#6-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ...

#DQ392963      TCA TGT CTA AGG AAG TGA AGC GTG TTG GTT ATG GCA ATG CAG ATA TCG GTA CCC AAG TCG ATA ATT TTT GGC TGG TGG
#AY750911      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AY750907      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AY750909      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519086      ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519088      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519089      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519090      .A. ..C AA. GT. ..T ..C .A. ..A ... ... ... ... ..A TGC .A. .G. .C. .GG ... .T. ..C .A. ... ..T ..A AA.
#AJ309734      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#1-OXA51       ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ...
#2-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#6-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#DQ392963      GTC CTT TAA AAA TTA CTC CTC AGC AAG AGG CAC AGT TTG CTT ACA AGC TAG CTA ATA AAA CGC TTC CAT TTA GCC AAA
#AY750911      ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#AY750907      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..
#AY750909      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519086      ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519088      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519089      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#DQ519090      .G. ... .G. C.. ... .A. ..A TA. ... .A. T.A ... ... TG. .TG .TT ... .CC .AG GGC AAT .G. .T. ... AA. CTG
#AJ309734      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..
#1-OXA51       ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ..G
#2-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..
#6-OXA51       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#DQ392963      AAG TCC AAG
#AY750911      ... ... ...
#AY750907      ... ... ...
#AY750909      ... ... ...
#DQ519086      ... ... ...
#DQ519088      ... ... ...
#DQ519089      ... ... ...
#DQ519090      ... .T. .GC
#AJ309734      ... ... ...
#1-OXA51       ... ... ...
#2-OXA51       ... ... ...
#6-OXA51       ... ... ...
```

APPENDIX F

EXAMPLES OF MHT, DDST AND CDT

Figure F.1. Positive MHT test of an isolate from this study. IMP disk at the centre of the plate. Clover leaf shaped zone indicates the presense of carbapenemase

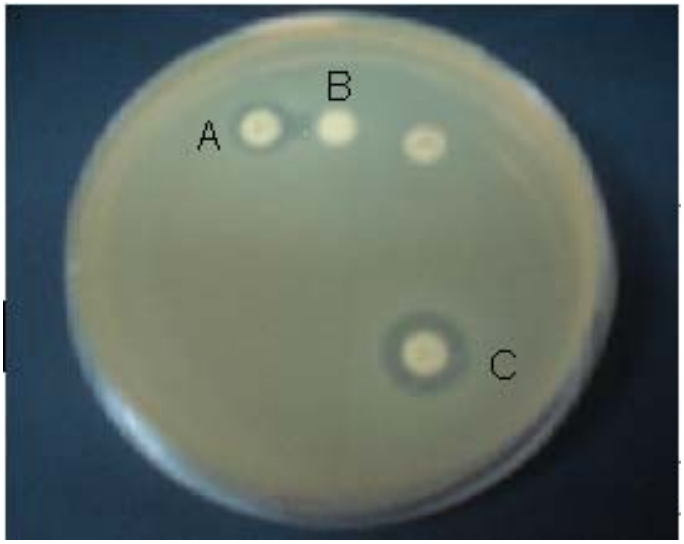

Figure F.2. Positive DDST and CDT of the positive control

A: IMP disk

B: Blank Disk with EDTA

C: IMP disk with EDTA

There is a synergy between A and B, and increased zone diameter in C

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PUBLICATIONS

Aylin Üsküdar Güçlü, Abdullah Kılıç, Ayten Küçükkaraaslan, Mehmet Baysallar, Levent Doğancı **"**Beyin omurilik sıvılarından izole edilen mikroorganizmalar ve antibiyotik duyarlılıkları" Gülhane Tıp Dergisi 2005; 47: 204-208.

Aylin USKUDAR GUCLU, Mehmet BAYSALLAR, Ayse Gul GOZEN, Abdullah KILIÇ, Arzu BALKAN, Levent DOĞANCI "Polymerase chain reaction vs. conventional culture in detection of bacterial pneumonia agents" 2005 Annals of Microbiology 55(4): 313-316.

Kilic Abdullah; Guclu Aylin Uskudar; Senses Zeynep; Bedir Orhan; Aydogan Hakan; Basustaoglu A Celal **"**Staphylococcal cassette chromosome mec (SCCmec) characterization and panton-valentine leukocidin gene occurrence for methicillin-resistant Staphylococcus aureus in Turkey, from 2003 to 2006" Antonie van Leeuwenhoek 2008;94(4):607-14.

Mehmet Baysallar, Aylin Üsküdar Güçlü, Zeynep Þenses, Kürþat Kaptan, Selmin Ataergin, Ahmet Celal Başustaoğu "Febril nötropenik hastaların kan kültürlerinde bakteriyel spektrum ve antimikrobiyal duyarlılık profili" Gülhane Tıp Dergisi 2007; 49: 168-172.

Ozgur Koru; Fulya Toksoy; Cengizhan Açıkel,Yaşar Meriç Tunca;Mehmet Baysallar; Aylin Üsküdar Güçlü; Eralp Akça; Aslı Özkök Tüylü; Kadriye Sorkun;Mehmet Tanyüksel;Bekir Salih "In Vitro Antimicrobial Activity of Propolis Samples from Different Geographical Origins Againts Certain Oral Pathogens" Anaerobe,17475517,7 Mar,2007.(SCI)

Senses, Zeynep; Yokusoglu, Mehmet; Oz, Bilgehan Savas; Guclu Aylin Uskudar; Erdem, Hakan; Baysan, O**.** "A rare pathogen causing endocarditis: Streptococcus constellatus" Anatolian Journal of Cardiology Aug 1, 2008, Letter to the editor.