THE INVESTIGATION OF OXACILLINASE / METALLO-BETA-LACTAMASE GENES AND CLONAL ANALYSIS IN CARBAPENEM RESISTANT *ACINETOBACTER BAUMANNII*

ARZU ALTUNÇEKIÇ YILDIRIM[1], YELIZ ÇETINKOL[2], MURAT TELLI[3]
[1]Ordu University Faculty of Medicine, Infectious Diseases Department, Ordu, Turkey - [2]Ordu University Faculty of Medicine, Medical Microbiology Department, Ordu, Turkey - [3]Adnan Menderes University Faculty of Medicine, Medical Microbiology Department, Aydin, Turkey

ABSTRACT

Introduction*: Acinetobacter baumannii isolates cause multidrug resistant infections and serious nosocomial outbreaks. The increase in resistance to a variety of antibiotics and carbapenems globally forms a serious clinical problem. In our study, we aimed to investigate the presence of oxacillinase and metallo-beta-lactamase genes responsible for resistance in carbapenem resistant A.baumannii isolates isolated in our hospital and to reveal the clonal relationship between these isolates.*

Materials and methods*: A.baumannii isolates of identification was completed using traditional methods and a fully automatic identification kit. Carbapenem-resistant isolates were evaluated using the minimal inhibition concentrations (MIC) E-test method. Oxacillinase and metallo-beta-lactamase genes were researched using polymerase chain reaction (PCR). Pulsed-field gel electrophoresis (PFGE) experiment was completed to determine the clonal relationship of carbapenem-resistant isolates.*

Results*: When the antibiotic susceptibility of A.baumannii isolates are assessed, the most effective antibiotic for all isolates was found to be colistin with 97.1% susceptibility rate. All carbapenem resistant isolates were found positive for OXA-51, with 97% positive for OXA-23, and 5.7% positive for OXA-24. One isolates was found to have the VIM resistant gene. None of the isolates were found to have OXA-58, OXA-48, IPM, SPM, SIM, GIM and NDM-1 genes. In the clonal distribution of isolates 3 different pulsotypes were determined. Of these 38 were a, 11 were b and 3 were c pulsotypes. The majority of isolates (73%) were shown to belong to a single clone and this was assessed as the outbreak isolate.*

Conclusion*: In our study, colistin was the most effective antibiotic against A.baumannii. OXA-23 was the most common carbapenemase among A. baumannii isolates in our hospital. Carbapenem-resistant A.baumannii strains producing OXA-23 have the potential for outbreak. Monitoring of resistance mechanisms is important to identify appropriate treatment approaches and to prevent the spread of resistant strains.*

Keywords*: Acinetobacter baumannii, carbapenem resistance, oxacillinase, metallo-beta-lactamase, clonal analysis, VIM.*

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Introduction

Acinetobacter baumannii isolates are pathogens that may have multiple drug resistance and cause severe nosocomial infections[1,2]. They may cause bacteraemia, secondary meningitis, urinary system infections and late onset nosocomial pneumonia, especially in intensive care units. Factors such as advanced age, serious underlying disease, immunosuppression, invasive interventions such as catheter insertion and mechanical ventilation, lengthened hospital stay and use of broad-spectrum antibiotics increase the incidence of infections linked to *A.baumannii*[3].

Acinetobacter have differing rates of antimicrobial susceptibility, with resistance frequently observed for beta-lactam antibiotics, aminoglycosides and fluoroquinolones. The inappropriate and widespread use of broad-spectrum cephalosporins, especially, has been found to be related to the occurrence of carbapenem resistant Acinetobacter spp. isolates[4]. Increasing resistance to carbapenem group antibiotics, chosen for infections developing due to multiple resistant isolates, is an issue in our country and the world[5].

Resistance in Acinetobacter type bacteria develops due to mechanisms such as production of modified enzymes of beta-lactamases and amino-

glycosides, changes in the target molecule and changes in outer membrane proteins[6]. The most frequent cause of carbapenem resistance in A.baumannii isolates is the presence of beta-lactamase. The most important beta-lactamases are D group series oxacillinases (OXA types) and less frequently carbapenemases including B group metallo-beta-lactamases (IMP, VIM, SIM, GIM, SPM metallo-beta-lactamases)(7). Within D group, generally naturally occurring OXA-51 and acquired OXA-23, OXA-24 and OXA58 subsets are important[8]. The majority of A.baumannii isolates containing these genes show resistance not just to carbapenems but to all other antibiotics in wide clinical use, apart from colistin and tigecycline, and cause infections with high mortality[9]. Resistance rates may show variation within countries, cities and even hospitals[10].

This study aimed to determine the antibiotic susceptibility of carbapenem-resistant A.baumannii isolates isolated at our hospital, to use molecular methods to identify the presence of oxacillinase and metallo-beta-lactamase gens causing carbapenem resistance and to reveal the clonal relationships between the isolates using "pulsed-field gel electrophoresis" (PFGE). We aimed to determine resistance mechanisms in Acinetobacter species that we isolate in our hospital. So we think that we can create the right antibiotic policies.

Materials and methods

Bacterial Isolates

Our study included 70 carbapenem-resistant A.baumannii isolates isolated as infection factors by the Ordu University Medical Microbiology Laboratory between January 2012 and January 2014. A single isolates from each patient was studied. *A.baumannii* ATCC 19606 isolates was used as control isolates.

Identification of Isolates

Stock isolates were revived by passaging on sheep's blood agar twice. For phenotypic species differentiation traditional methods and a fully-automated identification kit VITEK 2 (bioMerieux, France) were used according to the manufacturer's instructions.

Antibiotic Susceptibility Tests

All A.baumannii isolates were tested and interpreted for susceptibility to imipenem, meropenem, amikacin, netilmicin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole, gentamicin, levofloxacin, cefoperozone/sulbactam and colistin with the disk diffusion method according to the "Clinical and Laboratory Standards Institute (CLSI)" guidelines. For carbapenem-resistant isolates the minimal inhibitory concentrations (MIC) of imipenem, meropenem, amikacin, netilmicin, tetracycline, tigecylcine, trimethoprim/sulfamethoxazole, gentamicin, levofloxacin, cefoperozone/sulbactam and colistin were determined with the E-test method (Biomeriux, France) in accordance with the manufacturer's instructions and interpreted according to the CLSI guidelines[11]. Due to the lack of limit values in the CLSI guidelines, the susceptibility limit values for cefoperozone/sulbactam of <=16, 32, >=64 mg/L and for tigecycline of <=2, 4, >= 8 mg/L were taken from the prospectus information of the drug production firm.

Research of Carbapenem Resistance Mechanisms with Molecular Methods

The presence of oxacillinase genes of OXA-58 group, OXA-23 group, OXA-51 group, OXA-24 group and OXA-48 and of metallo-beta-lactamase genes of IPM, VIM, GIM, SPM, SIM and NDM-1 were investigated using the specific gene sequences (Table 1) coding these enzymes in the gene region using polymerase chain reaction (PCR). Bacterial cell preparation and PCR for antibiotic resistance

Primer Name	Gene Sequence
OXA23-F	GAT CGG ATT GGA GAA CCA GA
OXA23-R	ATT TCT GAC CGC ATT TCC AT
OXA24-F	GGT TAG TTG GCC CCC TTA AA
OXA24-R	AGT TGA GCG AAA AGG GGA TT
OXA51-F	TAA TGC TTT GAT CGG CCT TG
OXA51-R	TGG ATT GCA CTT CAT CTT GG
OXA58-F	AAG TAT TGG GGC TTG TGC TG
OXA58-R	CCC CTC TGC GCT CTA CAT AC
IMP F	GGA ATA GAG TGG CTT AAY TCT C
IMP-R	CCA AAC YAC TAS GTT ATC T
VIM- F	GAT GGT GTT TGG TCG CAT A
VIM-R	CGA ATG CGC AGC ACC AG
GIM-1-F	TCG ACA CAC CTT GGT CTG AA
GIM-1-R	AAC TTC CAA CTT TGC CAT GC
SPM-1- F	AAA ATC TGG GTA CGC AAA CG
SPM-1- R	ACA TTA TCC GCT GGA ACA GG
SIM-1- F	TAC AAG GGA TTC GGC ATC G
SIM-1- R	TAA TGG CCT GTT CCC ATG TG
NDM-1 F	CCA ATA TTA TGC ACC CGG TCG
NDM-1 R	ATG CGG GCC GTA TGA GTG ATT G

Table 1: Primary gene sequences used in multiplex PCR.

genes was performed as previously described by Park et al[12]. Identified VIM 2 was confirmed with sequence results.

Research of Clonal Relationships

The PFGE experiment was carried out to research the clonal relationship of carbapenem-resistant isolates[13].

PFGE

Pure A.baumannii isolates and 1 ml cell suspension solution (HSS; 100M Tris-HCL, 100mM EDTA, pH 8.0) with stirring, 4 McFarland bacterial suspension was prepared. Bacterial suspension was centrifuged at 13,000 rpm for 2 minutes. The supernatant was discarded and pellet was dissolved with 1 ml HSS. Of this solution 100 μl was mixed with 5 μl proteinase K (20 mg/kg stock). Low melting agar (LMA) of 2% including 1% sodium dodecyl sulfate was mixed with 100 μl of this bacterial suspension. Later this mixture was distributed to a plug-mold. The formed bacterial gel plates were placed in a liquid containing 1 ml cell lysis solution (CLS; 50%mM Tris, 50%mM EDTA, pH 8, 1% sarkosyl) and 3 μl proteinase K (20 mg/ml stock) and incubated for 2 hours at 54 °C. Later a washing procedure with distilled water twice, and TE buffer 4 times at 50 °C was performed. At the end of lysisthe DNA was cut with ApaI (30 units) enzyme. The cut DNA fragments were put through a CHEF DR 3 (Bio-Rad) system within 1% agarosecel (initial 5 s, final 30 s, degree 120°, 6 V/cm2, 20 hours). The bands formed were imaged in UV light for 40 minutes in a 15 μl/200 ml safeview. The PFGE images were analyzed with Bio1D software (Vilber Lourmart) and the dendograms were obtained. PFGE relationship was evaluated as the same genotype for those with ≥80%. Within the same genotype 3 or more band differences observed was accepted as a subtype[14].

Statistical Analysis

SPSS version 22 (SPSS Inc, Chicago, IL) was used for statistical analysis. Categorical variables are stated as percentages.

Results

The study included 70 A.baumannii isolates obtained from a variety of clinical samples belonging to 44 men and 26 women. The isolates isolated from intensive care included 51 respiratory tract samples (72.9%), 11 blood samples (15.7%), 5 urine samples (7.1%) and 3 abscess samples (4.3%). The mean age of patients with A.baumannii isolated was found to be 67.2 years.

When the antibiotic susceptibility of A.baumannii isolates is evaluated, the most effective antibiotic for all isolates was colistin with 97.1% susceptibility rates. When the colistin resistant isolates were re-evaluated with the E-test method, the MIC values were found to be >16 µg/ml. When the other antibiotic susceptibilities of these isolates with multiple drug resistance are investigated, 68.6% were susceptible to trimethoprim/sulfamethoxazole and 21.4% were susceptible to tigecycline. The susceptibility rates of A.baumannii isolates against all tested antibiotics are given in Table 2.

Antibiotic	Interval	MIC 50	MIC 90	Susceptibility Amount (%)	Moderate Susceptibility Amount (%)	Resistance Amount (%)
Amikacin	<=16, 32, >=64	>128	>128	6 (8.6%)	4 (5.7%)	60(85.7%)
Netilmicin	<=8, 16, >=32	>64	>64	9 (12.9%)	9 (12.9%)	52(74.2%)
Tetracycline	<=4, 8, >=16	16	64	10(14.3%)	-	60(85.7%)
Tigecycline	<=2, 4, >=8	4	8	15(21.4%)	22(31.4%)	33(47.1%)
Trimethoprim/Sulfamethoxazole	<=2/38,-,>=4/76	2	4	48(68.6%)	-	22(31.4%)
Gentamicin	<=4, 8, >=16	16	32	7 (10.0%)	-	63(90.0%)
Levofloxacin	<=2, 4, >=8	8	32	3 (4.3%)	5 (7.1%)	62(88.6%)
Cefoperazone/Sulbactam	<=16, 32, >=64	32	256	7 (10.0%)	10 (14.3%)	53(75.7%)
Piperacillin/Tazobactam	<=16/4, 32/4-64/4, >=128/4	>256	>256	2 (2.9%)	1(1.4%)	67(95.7%)
Ceftazidime	<=8, 16, >=32	>64	>64	2 (2.9%)	1(1.4%)	67(95.7%)
Colistin	<=2, -, >=4	0,5	2	68(97.1%)	-	2 (2.9%)

Table 2: The susceptibility rates of A.baumannii isolates against all tested antibiotics (n=70).

All of these carbapenem-resistant isolates were positive for OXA-51, with 68 positive for OXA-23 (97%) and 4 positive for OXA-24 (5.7%) (Figure 1). Of metallo-betalactamases, in only 1 isolates (1.4%) was the VIM resistance gene encountered. In none of the isolates were OXA-58, OXA-48, IPM, SPM, SIM, GIM and NDM-1 genes found.

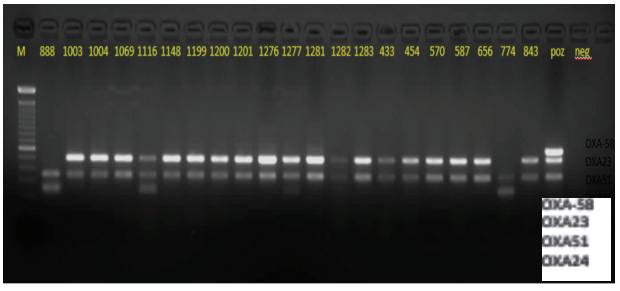

Fig. 1: Identification of genes coding OXA-carbapenemase with multiplex PCR.

When the results of molecular typing are examined, PFGE determined 3 separate pulsotypes in the clonal distribution of 52 isolates. These were shown to be 39 a (75%), 11 b (19%) and 3 c

(6%) pulsotypes. When the pulsotype distribution is evaluated according to whether isolates carry OXA genes, 38 of the isolates carrying OXA-23 gene were a, 11 isolates were b and 1 isolates was c pulsotype. Two isolates not carrying OXA-23 were shown to be c pulsotype. When examined in terms of OXA-24 gene, 3 isolates carrying this gene were c pulsotype and 1 isolates was a pulsotype, while 37 isolates not carrying this gene were a and 11 isolates were b pulsotype. When the similarity between isolates with these pulsotypes is investigated, the majority of the isolates (75%) were shown to belong to a single clone and this was evaluated as the outbreak isolate (Figure 2).

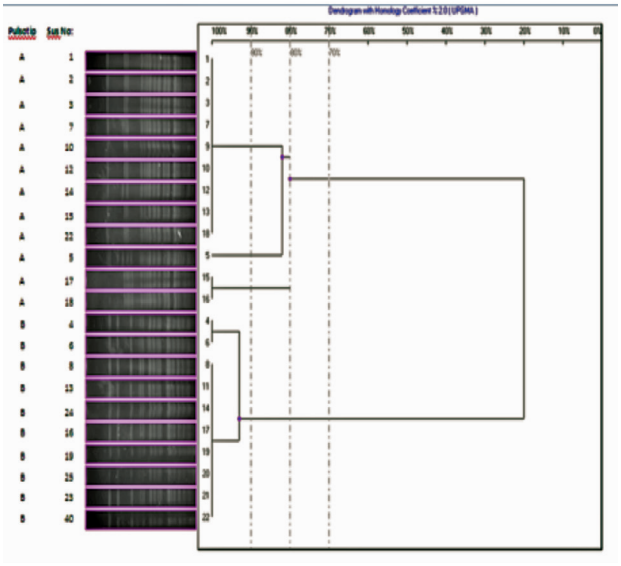

Fig. 2: Dendogram of most common pulsotypes of selected strains.

Discussion

For one of the significant factors in nosocomial infections, *A.baumannii* isolates, carbapenem resistance is an important problem limiting treatment choices[15]. Though carbapenem resistance of *A.baumannii* occurs by more than one mechanism, resistance most frequently occurs though beta-lactamase enzyme pathways. The majority of *A.baumannii* isolates producing beta-lactamase are resistant, not just to carbapenems but to all other antibiotics widely used in clinical practice, apart from colistin and tigecycline[16]. The last choice for carbapenem resistance isolates is colistin and there are reports of an increase in resistance to polymyxin B[17]. A variety of studies in our country have reported carbapenem resistance varies between 33% and 96%[18,19].

Different studies with other antibiotics have observed varying resistance rates of 41-70% for amikacin, 32-87% for ciprofloxacin, 46-93.3% for cefepime, 62-87% for gentamicin, 63-75% for trimethoprim/sulfamethoxazole , 44.1-100% for piperacillin/tazobactam, 77-85% for ceftriaxone and 65-92.5% for ampicillin/sulbactam[20,21].

In our study the most effective antibiotic against A.baumannii isolates was identified as colistin with 97.1% susceptibility. The antibiotic susceptibility rates for trimethoprim/sulfamethoxazole, tigecycline, tetracycline, netilmicin, gentamicin, cefoperozone/sulbactam, levofloxacin, piperacillin/tazobactam and ceftazidime were 68.6%, 21.4%, 14.3%, 12.9%, 10.0%, 10.0%, 4.3%, 2.9% and 2.9%, respectively. The results we obtained appear to be in accordance with other studies in our country.

In our study the presence of oxacillinase and metallo-beta-lactamase genes causing carbapenem resistance was researched with PCR. While all isolates were positive for OXA-51 gene, 97% were positive for OXA-23 gene and 5.7% were positive for OXA-24 gene. None of the isolates were identified to have OXA-58, OXA-48, IPM, SPM, SIM, GIM and NDM-1 genes. In only a single isolates (1.4%) was the VIM resistant gene identified. The presence of the OXA-51 gene, specific to the A.baumannii species in all isolates was confirmed by molecular differentiation of isolates included in the study. When other studies in our country are investigated, while Külah et al.[22] found the OXA-58 gene was dominant they did not identifiy the OXA-23 and OXA-24 genes. Some studies have identified different rates of OXA-23 and OXA-58 genes, the OXA-23 gene especially is dominant and it is stated that the incidence rate has increased in recent times[5,23,24].

Additionally in a study of 834 A.baumannii isolates isolated from different regions in our country Çiftçi et al.[5] found 53.7% positivity for OXA-23 gene. When various studies from this country and abroad are evaluated, positivity rates of OXA-23 are reported to vary from 0-98.4%[24-26]. In our study while OXA-23 was dominant, different to these studies, resistant gene positivity for VIM was found in one isolates. In our country VIM gene presence in Acinetobacter isolates was only found in one study. A study by Sarıgüzel et al.[27] researched IPM-1 and VIM-1 genes in A.baumannii isolates isolated from blood cultures and showed VIM-1 gene in 3 isolates.

In our study VIM 2 was identified and confirmed with sequence results. So this result may be considered the first report of VIM-2 gene in our country. In the world in general VIM gene is reported in very low numbers from A.baumannii isolates. The first reports were of VIM-2 from South Korea and VIM-1 from Greece. In Europe, especially in Mediterranean countries, there are limited reports of Acinetobacter isolates carrying these genes, while it appears to be endemic in some Asian countries[17]. In recent times VIM resistance genes with rates varying from 1% to 85% have been reported by different studies in Saudi Arabia, India, Egypt and South Africa. MBL production is related to high levels of carbapenem resistance, aminoglycoside and fluoroquinolone resistance and resistance to beta lactams apart from aztreonam. This situation limits treatment choices to polymyxins[28-31].

High rates of multiple resistant isolates indicate increasing spread of resistance between bacteria and possibly clonal distribution. Currently the "gold standard" in outbreak research, the PFGE method was used to evaluate clonal distribution of isolates and 3 separate pulsotypes of 73.1% a, 21.1% b and 5.8% c pulsotype were determined. In our study 2 A.baumannii isolates not carrying OXA-23 and 3 of the 4 isolates carrying OXA-24 gene were observed to be c pulsotype. Additionally the isolates with VIM-2 resistant gene was observed to be b pulsotype. In our country in a study by Özen et al.[32] in 2009 an outbreak linked to A.baumannii carrying OXA-58 gene and with multiple drug resistance was shown to have been sourced from a single clone by PFGE. Again a study by Keskin et al. found in 2010 isolates from c and d genotypes were dominant while they reported that in 2011 a and b genotypes had become dominant[33].

The resistance rates of A.baumannii isolates frequently isolated as an infection factor in recent years is increasing, and the increasing trend for colistin resistance, especially, requires careful monitoring of antibiotic susceptibilities and resistance profiles. In our hospital, similar to the world in general, A.baumannii isolates are identified to have high rates of OXA group genes. Additionally of MBL genes VIM-2 gene presence was shown, not previously reported in our country.

In conclusion, to understand the spread of isolates with these genes, broad scale and multicenter epidemiological studies are required.

Additionally to determine effective treatment methods and control the spread of resistance, we believe it is necessary to develop simple and cheap screening methods to rapidly determine the presence of oxacillinase/metallo-beta-lactamase genes.

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Corresponding Author:
Arzu Altunçekiç Yildirim, MD.
Ordu Üniversitesi Eğitim ve Araştırma Hastanesi, Bucak Mah. NefsiBucak Cad. 52200, Ordu,
e-mail: arzu_al@yahoo.com
(Turkey)