Balkan Med J 2015;32:79-83

Investigation of Metallo Beta Lactamases and Oxacilinases in Carbapenem Resistant *Acinetobacter baumannii* Strains Isolated from Inpatients

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Background: Resistance to beta-lactam antibiotics is widespread among *Acinetobacter* strains. Plasmid-mediated metallo beta lactamases (MBL) are responsible for carbapenem resistance, as are oxacillinases (OXA). In recent years, MBL producing carbapenem-resistant strains have been reported in the world and in Turkey in increasing rates. In our country, besides the OXA 51-like enzyme which is inherent in *A. baumannii* strains, OXA 58-like and OXA 23-like carbapenemases producing strains have also been widely detected. In addition, Verona Imipenemase (VIM) and (IMP)-type MBL have been reported in some centers.

Aims: The aim of our study was to investigate the presence of carbapenemases in *Acinetobacter* strains isolated from hospitalized patients in Edirne.

Study Design: Cross-sectional study.

Methods: A total of 52 imipenem-resistant A. baumannii strains isolated between January and March 2013 were investigated. The presence of MBL was described phenotypically by the combined disk diffusion test (CDDT), double disk synergy test (DDST), MBL E-test (only performed in 28 strains) and modified Hodge test. bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, bla_{SPM} genes and bla_{OXA-23}, bla_{OXA-51}, bla_{OXA-40}, bla_{OXA-58} genes were investigated by multiplex polymerase chain reaction (PCR). The blaNDM-1 gene was determined by PCR.

Results: By modified Hodge test, 50 strains (96%) were found to be MBL positive. Positivity of MBL was 21% by both CDDT (0.1 M EDTA) and DDST. Twenty-four of 28 strains (85.7%) were positive by MBL E-test. OXA 23-like and OXA 51-like carbapenemases were detected in all strains, but OXA 58-like and OXA 40-like carbapenemases-producing *A. baumannii* were not detected. Also, MBL genes were not detected by genotypic methods.

Conclusion: Only OXA 23-like carbapenemase was responsible for carbapenem resistance in carbapenem-resistant *Acinetobacter* strains in Edirne. The MBL-producing *Acinetobacter* strain is not yet a problem in our hospital. MBL resistance was found by phenotyping tests, which must be confirmed by genotypic methods; multiplex PCR tests can be easily used for screening MBL.

Keywords: *Acinetobacter baumannii*, metallo beta lactamases, oxacillinases

Acinetobacter baumannii strains are non-fermenting aerobic Gram-negative bacteria that play an important role in hospital infections. Difficulties in the treatment and control of this growing threat make this pathogen an important health problem (1). Resistance to beta-lactam antibiotics is widespread among *Acinetobacter* strains. Plasmid-mediated metallo beta-lactamases

This study was presented as a poster at the 53rd ICAAC Congress, 10-13 September 2013, Denver, Colorado, USA.

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Received: 30.12.2013 Accepted: 30.11.2014 • DOI: 10.5152/balkanmedj.2015.15302
Available at www.balkanmedicaljournal.org

Cite this article as:
Aksoy MD, Çavuşlu Ş, Tuğrul HM. Investigation of metallo beta lactamases and oxacilinases in carbapenem resistant *acinetobacter baumannii* strains isolated from inpatients.
Balkan Med J 2015;32:79-83.

(MBL) are responsible for carbapenem resistance, as well oxacillinases (OXA). In recent years, MBL producing carbapenem-resistant strains have been reported in the world and in Turkey in increasing rates (2). OXA-51-like, OXA-58-like, OXA-23-like carbapenemase-producing strains were detected widely (3, 4). Also, in a recent study, it was reported that a small outbreak was caused by *A. baumannii* which produced OXA-72, a member of OXA-24/40-like enzymes in a Turkish hospital (5). Four types of MBL enzymes have been identified in *A. baumannii* isolates including Imipenemase (IMP), Verona Imipenemase (VIM), Seoul Imipenemase (SIM) and New Delhi Metallo beta-lactamase (NDM)-1-types throughout the World (6). VIM, IMP and NDM-1-type MBL enzymes have been reported in some centers in Turkey (7-10). In this study, the presence of carbapenemases was investigated by phenotypic methods and the responsible resistance genes were investigated by polymerase chain reaction (PCR) and multiplex PCR in *Acinetobacter* strains, isolated from hospitalized patients in Edirne.

MATERIALS AND METHODS

Strain selection

Between January and March 2013, a total of 52 imipenem-resistant *A. baumannii* strains were isolated from clinical samples (endotracheal aspirates, sputum, blood, urine, intravenous catheter, aspirate, wound, tissue, and cerebrospinal fluid) of different patients. Identification and antibiotic susceptibility analysis of the strains were performed by VITEK 2 system (bioMerieux, Marcy l'Etoile, France). Strains were stored at -80°C until the other tests were performed. *P. aeruginosa* ATCC 27853 was used as a negative control strain. MBL-producing *P. aeruginosa* and carbapenemase-producing *A. baumannii* were used as positive control strains. The positive control strains with a known content of carbapenemases OXA, MBL enzymes) were provided from the Department of Microbiology, Istanbul Faculty of Medicine, İstanbul University in Turkey.

Phenotypic determination of carbapenemase production

The presence of MBL was described phenotypically by the combined disk diffusion test (CDDT) with 0.1 M and 0.5 M ethylenediamine-tetraacetic acid (EDTA), double disk synergy test (DDST), and MBL E-test (only performed in 28 strains) and the presence of all carbapenemases was described by the modified Hodge test (MHT).

Combined disk diffusion test: Imipenem (10 mg) disks, imipenem/0.1 M EDTA and imipenem/0.5M EDTA combined disks were used for screening. Increases of ≥4 mm in the zone diameters of imipenem disks in the presence of 0.1 M EDTA and Increases of ≥7 mm in the zone diameters of imipenem disks in the presence of 0.5 M EDTA were interpreted as a possible MBL positivity (11).

Double disk synergy test: Imipenem disks were placed 20 mm (center to center) away from the blank disk. Then, 10 mL of 0.5 M EDTA were added on the blank disk. Zone enhancement between two disks was evaluated positive for MBL (12).

MBL E-test: The MBL E-test strip (bioMerieux, Solna, Sweden) containing a double sided of Imipenem (4 to 256 µg/ml) and Imipenem (1 to 64 µg/ml) in combination with a fixed concentration of EDTA was used for MBL detection. It was evaluated according to the instructions. MIC ratio of ≥8 for the 2 reagent sides of imipenem and imipenem with EDTA was indicative MBL production This test was only performed in 28 strains (13).

Modified Hodge test: A 0.5 McFarland dilution of *Escherichia coli* ATCC 25922 was prepared. A 1:10 dilution was streaked as a lawn onto a Mueller Hinton Agar (MHA) plate (Merck, Darmstadt, Germany) and a 10 µg imipenem disk was placed in the center of the plate. *A. baumannii* strains were streaked in a straight line from the edge of the disk to the edge of the plate. After 24 hours, if test organisms had carbapenemases, the test showed a cloverleaf-like indentation of the *E. coli* growing along the test organism growth streak within the disk diffusion zone (12).

Genotypic determination of carbapenemase production

Strains selected for gene detection were inoculated onto MHA at 37°C for 18 hours. One colony from this fresh culture was resuspended in 200 µL of sterile water and heated at 95°C for 10 minutes. This was followed by centrifugation at 13,000×g for 10 minutes. The obtained supernatant was served for PCR using the Thermocycler (iCycler iQ Real Time PCR, Minnesota, USA) with the specific primers shown in Table 1.

Responsible resistance genes were investigated by PCR and multiplex PCR in *Acinetobacter* strains. The $bla_{\text{IMP- like}}$, $bla_{\text{VIM-like}}$, $bla_{\text{GIM-1}}$, $bla_{\text{SIM-1}}$, $bla_{\text{SPM-1}}$ genes and $bla_{\text{OXA-23,}}$ $bla_{\text{OXA-51,}}$ $bla_{\text{OXA-40}}$, $bla_{\text{OXA-58}}$ genes were investigated by multiplex PCR (14, 15); these PCRs were run as two separate tubes. The $bla_{\text{NDM-1}}$ gene was determined by individual PCR (16).

The final 50 µL PCR mixture contained 10x PCR buffer (10 µL), 2 mM deoxynucleoside triphosphates (dNTP) (10 µL), 2.5 mM $MgCI_2$ (5 µL), each of the forward and reverse primers (1 µL), 1 U Taq DNA polymerase (2 µL) and genomic DNA of the test strain (2 µL). Thermal cycling was programmed for multiplex PCR at 94°C for 5 min, followed by 30 cycles of amplification. Each cycle consisted of 94°C for 25 s, 52°C for 40 s, and 72°C for 50 s. A final extension step (72°C for 6 min) completed the amplification (15). Then, single NDM-1

TABLE 1. The primers of target genes used for PCR

Primer	Sequence (5'-3')	Product size (bp)	Reference
Bla_{IMP}-F	5'-GGAATAGAGTGGCTTAATTCTC	(188 bp)	
Bla_{IMP}-R	5'-CCAAACCACTACGTTATCT		
Bla_{SPM-1}-F	5'-AAAATCTGGGTACGCAAACG	(271 bp)	
Bla_{SPM-1}-R	5'-ACATTATCCGCTGGAACAGG		
Bla_{VIM}-F	5'-GATGGTGTTTGGTCGCATA	(390 bp)	
Bla_{VIM}-R	5'-CGAATGCGCAGCACCAG		
Bla_{GIM}-1-F	5'-TCGACACACCTTGGTCTGAA	(477 bp)	
Bla_{GIM}-1-R	5'-AACTTCCAACTTTGCCATGC		
Bla_{SIM}-1-F	5'-TACAAGGGATTCGGCATCG	(570 bp)	Ellington et al. 2007 Woodford 2010
Bla_{SIM}-1-R	5'-TAATGGCCTGTTCCCATGTG		
Bla_{OXA-51}-F	5'-TAA TGC TTT GAT CGG CCT TG	(353 bp)	
Bla_{OXA-51}-R	5'-TGG ATT GCA CTT CAT CTT GG		
Bla_{OXA-23}-F	5'-GAT CGG ATT GGA GAA CCA GA	(501 bp)	
Bla_{OXA-23}-R	5'-ATT TCT GAC CGC ATT TCC AT		
Bla_{OXA-40}-F	5'-GGT TAG TTG GCC CCC TTA AA	(246 bp)	
Bla_{OXA-40}-R	5'-AGT TGA GCG AAA AGG GGA TT		
Bla_{OXA-58}-F	5'-AAG TAT TGG GGC TTG TGC TG	(599 bp)	
Bla_{OXA-58}-R	5'-CCC CTC TGC GCT CTA CAT AC		
Bla_{NDM-1}-F	5'-ACC GCC TGG ACC GAT GAC CA	(264 bp)	Zarfel et al. 2011
Bla_{NDM-1}-R	5'-GCC AAA GTT GGG CGC GGT TG		

IMP: imipenemase; SPM: Sao Paulo Metallo-beta-lactamase; VIM: verona Imipenemase; GIM: German imipenemase; SIM: Seoul imipenemase; OXA: oxacillinase; NDM: New Delhi metallo beta-lactamase

PCR at 94°C for 5 min was programmed, followed by 35 cycles of amplification. Each cycle consisted of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. A final extension step (72°C for 10 min) completed the amplification (16). PCR products were analyzed by electrophoresis in a 2% agarose gel containing 0.5 mg/L ethidium bromide at 100V for 60 minutes and compared with the 100 bp DNA Ladder (Fermentas, St. Leon-Rot, Germany).

RESULTS

A total of 52 carbapenem-resistant *A. baumannii* strains were investigated. These strains had been collected from intensive care units (ICUs) (62%), internal medicine clinics (23%) and surgical clinics (15%). Most of the 52 strains of *A. baumannii* were isolated from respiratory samples (endotracheal aspirates, sputum) (54%). 50 strains (96%) were detected as carbapenemase positive by the modified Hodge test. Positivity of MBL was found to be 21%, 21% and 98% by CDDT (0.1 M EDTA), DDST and CDDT (0.5 M EDTA), respectively. In total, 24 of 28 strains (85.7%) were found to be MBL positive by the E-test. OXA 23-like (100%) and OXA 51-like (100%) carbapenemases were detected in all strains, but OXA 58-like and OXA 40-like carbapenemases producing *A. baumannii* were not detected (Figure 1). Also, MBL genes were not detected by genotypic methods (Table 2).

DISCUSSION

Acinetobacter baumannii is an important opportunistic bacterial pathogen responsible for serious infections. They are predominant pathogens in ICUs in Turkey (17). In this study, most of the 52 strains of *A. baumannii* were collected from ICUs (62%). Carbapenem resistance was investigated by phenotypic and genotypic tests of the strains. Although some *A. baumannii* strains were found to be MBL positive by phenotypic tests which were established for EDTA-susceptible and EDTA-non-susceptible strains, MBL resistance genes were not detected. There was a discrepancy between the phenotypic and genotypic results. It was thought that the EDTA might be responsible for the false MBL positivity in phenotypic tests. Other studies have revealed that the membrane permeabilizing effect of EDTA can increase the susceptibility of Gram-negative bacteria such as *A. baumannii, P. aeruginosa* (18, 19). In our country, although Aktaş and Kayacan (11) detected some *Pseudomonas* and *Acinetobacter* strains to be MBL-positive by phenotypic tests, no isolate was found to be positive for the presence of bla_{IMP} or bla_{VIM} genes. Different studies have reported positive results by MBL phenotypic tests, but MBL resistance genes that are common in our country could not be identified in *Acinetobacter* strains (20-22). Rodrigues et al. (23) reported that the phenotypic test to detect

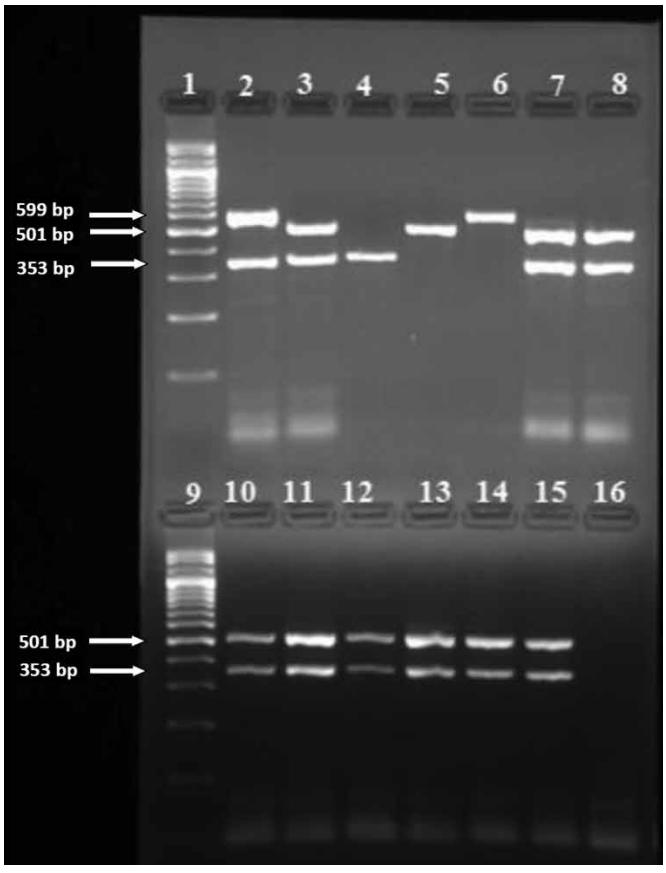

FIG. 1. Multiplex PCR gel showing of bla_{OXA-23} and bla_{OXA-51} positive *Acinetobacter* strains 1, 9: ladder (100bp); 2-6: positive controls (2: OXA-51, 58; 3: OXA-51, 23; 4: OXA-51; 5: OXA-23; 6: OXA-58), 7-15: clinical samples, 16: negative control

MBL was positive in 5% (2/40) of *P. aeruginosa* isolates; however, none of the investigated genes (bla_{IMP}, bla_{VIM}, bla_{SPM}) were identified by PCR. Ratkai et al. (24) reported that most *P. aeruginosa* strains were found to be MBL-positive by the MBL E test and combined disk test, but no MBL genes were detected. Chu et al. (25) reported false positive results in *P. aeruginosa* strains by MBL E-test and disk synergy methods using EDTA. MBL genes were not detected by PCR. As can be seen from the studies, carbapenem resistance genes, which are common in the region, should be investigated to evaluate the phenotypic test results correctly.

Although MBL resistance genes were not detected, other than the bla_{OXA-51} gene which is isolated in *A. baumannii* strains, the bla_{OXA-23} gene, which seems to responsible for the carbapenem resistance in Edirne, was detected in all of the strains tested in our study. These carbapenemases are particularly widespread in our country. OXA-23-like, OXA-51-like, OXA-58-like, OXA-24/40-like enzyme-producing *A. baumannii* strains were previously reported in Turkey (3-5, 26, 27). In Greece, Pournaras et al. (28) reported that OXA-51-like and OXA-58-like enzyme-producing *A. baumannii* strains were detected, but MBL genes (bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{SPM} genes) were not detected by PCR. In 2010-2011, Liakopoulos et al. (29) reported that OXA-23-like and OXA-58-like carbapenemases were detected in carbapenem-resistant *A. baumannii* isolates. Strateva et al. (30) reported OXA-23-like carbapenemase in multidrug-resistant *A. baumannii* isolates in a Bulgarian university hospital. Although OXA 58-like and OXA-24/40-like carbapenemases have been reported in neighboring countries and in Turkey, they were not detected in Edirne.

TABLE 2. Positivity and negativity rates of MBL by phenotypic and genotypic tests

	Acinetobacter baumannii (n=52)	
Phenotypic tests	MBL (+) n (%)	MBL (-) n (%)
CDDT		
IPM + 0.1 M EDTA	11 (21.1)	41 (78.8)
IPM + 0.5 M EDTA	51 (98)	1 (1.9)
DDST	11 (21.1)	41 (78.8)
MHT	50 (96)	2 (3.8)
MBL E Test	24 (85.7)	4 (14.2)
Genotypic tests		
MBL PCR	0	52
OXA PCR	52	0

MBL: metallo beta-lactamase; CDDT: combined disk diffusion test; EDTA: ethylenediamine-tetraacetic acid; DDST: double disk synergy test; MHT: modified hodge test; OXA: oxacillinase, PCR: polymerase chain reaction

As a conclusion, this study showed that only OXA 23-like carbapenemase was responsible for carbapenem resistance in carbapenem-resistant *Acinetobacter* strains in Edirne. MBL-producing *Acinetobacter* strains are not currently a problem in our hospital. MBL resistance found by phenotyping tests must be confirmed by genotypic methods; multiplex PCR tests can be easily used for screening MBL.

Ethics Committee Approval: N/A.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - M.D.A., H.M.T.; Design - M.D.A.; Supervision - Ş.Ç., H.M.T.; Resource - M.D.A, H.M.T.; Materials - M.D.A.; Data Collection&/or Processing - M.D.A, H.M.T.; Analysis&/or Interpretation - M.D.A.; Literature Search - M.D.A, Ş.Ç.; Writing - M.D.A.; Critical Reviews - Ş.Ç., H.M.T.

Acknowledgements: We thank Zerrin Aktaş from the Department of Microbiology, Istanbul Faculty of Medicine, Istanbul University in Turkey for granting the VIM-1, VIM-2, IMP-1, SPM-1, NDM-1, OXA-23, OXA-51, OXA-58 positive strains.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

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