Beta-lactamase Enzymes of Clinical *Pseudomonas aeruginosa* Strains

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ABSTRACT

Objectives: *In this study, the production of extended spectrum beta-lactamase (ESBL), metallo-beta-lacatamase (MBL) and AmpC beta-lactamase enzymes of* Pseudomonas aeruginosa (P aeruginosa) *strains which were isolated from clinical samples were investigated.* AmpC *gene was also detected by the polymerase chain reaction (PCR) analysis.*

Methods: *A hundred strains of* P aeruginosa *were included in the study. The presence of ESBL was investigated with combined disk confirmation test, MBL was investigated with E-test method and AmpC beta-lactamase was investigated with disk induction test. In order to detect the production of AmpC beta-lactamase genotypically, the PCR method was used.*

Results: *Only one strain was found to be MBL positive. Four per cent of strains were found to be ESBL positive. AmpC beta-lactamase production was positive in 73% of the strains with disk induction test.* AmpC *gene was detected in 96% of the studied strains with the PCR method.*

Conclusion: *While ESBL and MBL rates in this study were significantly lower than those found in other studies, the rate of AmpC beta-lactamase was higher. Although* AmpC *gene was detected in some strains (23%), they were not found to produce AmpC beta-lactamase with disk induction test*

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Keywords: AmpC beta-lactamase, *AmpC* gene, extended spectrum beta-lactamase, metallo-beta-lactamase, *Pseudomonas aeruginosa*, resistance

Las Enzimas Beta-lactamasas de Cepas Clínicas de *Pseudomonas aeruginosa*

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RESUMEN

Objetivos: *En este estudio, se investigó la producción de enzimas beta-lactamasa de espectro extendido (BLEE), metalo-beta-lactamasa (MBL), y beta-lactamasa tipo AmpC de cepas de la* Pseudomonas aeruginosa (P aeruginosa), *que fueron aisladas en muestras clínicas. El gen AmpC también fue detectado mediante análisis de la reacción en cadena de la polimerasa (RCP).*

Métodos: *Un centenar de cepas de* P aeruginosa *se incluyeron en el estudio. La presencia de BLEE fue investigada y confirmada mediante la prueba de discos combinados. La MBL fue investigada mediante el método E-test, y la beta-lactamasa tipo* AmpC *fue investigada mediante la prueba de inducción de disco. Para detectar la producción de beta-lactamasa tipo* AmpC *genotípicamente, se utilizó el método RCP.*

Resultados: *Sólo una cepa resultó ser MBL positiva. El cuatro por ciento de las cepas resultaron ser BLEE positivas. La producción de beta-lactamasa tipo* AmpC *fue positiva en el 73% de las cepas con la prueba de inducción de disco. El gen* AmpC *fue detectado en el 96% de las cepas estudiadas con el método RCP.*

Conclusión: *Si bien las tasas de BLEE y MBL en este estudio fueron significativamente inferiores a las encontradas en otros estudios, la tasa de beta-lactamasa tipo AmpC fue más alta. Aunque se detectó el gen* AmpC *en algunas cepas (23%), no se encontró que estas produjeran beta-lactamasa tipo* AmpC *con la prueba de inducción de disco.*

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Palabras claves: Beta-lactamasa tipo AmpC, gen AmpC, beta-lactamasa de espectro extendido, metalo-beta-lactamasa, *Pseudomonas aeruginosa*, resistencia

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INTRODUCTION

Production of beta-lactamase is one of the most important resistance mechanisms of many bacteria. Beta-lactamases inactivate the beta-lactam antibiotics by hydrolyzing them. One hundred and fifty of these beta-lactamase enzymes are extended spectrum beta-lactamases [ESBLs] (1, 2). Extended spectrum beta-lactamases hydrolyze penicillins and first, second and third generation cephalosporins. But carbapenems and cefamicins are stable against the hydrolyzing effects (2). Difficulties were encountered in the treatment of infections caused by ESBL-producing bacteria as they are generally multidrug resistant (3).

Metallo-beta-lactamases (MBLs) hydrolyze many beta-lactam antibiotics, and are not inhibited by beta-lactamase inhibitors. So the bacterial pathogens carrying acquired MBL genes show broad-spectrum resistance against beta-lactams.

Therefore, because of carbapenemase activities of these enzymes, MBL-producing bacteria affect carbapenems which are the drugs of last resort in the treatment of infections. Also, MBL genes are available with resistance genes of the other drugs; MBL-producing bacteria additionally exhibit resistance phenotype to some drugs (1, 4, 5).

Pseudomonas aeruginosa (*P aeruginosa*) strains carry inducible AmpC cephalosporinase similar to chromosomally encoded AmpC beta-lactamase that is present in a few *Enterobactericeae* members (6). *P aeruginosa* strains generally produce a low level of AmpC beta-lactamase and are sensitive to antipseudomonal penicillins, penicillin-inhibitor combinations, cephalosporins and carbapenems (7). However, when AmpC beta-lactamase production increases significantly, the resistance against all beta-lactams except carbapenem develops (7). The resistance of *P aeruginosa* emerging during treatment of a patient is a greater danger. Treatment with an appropriate beta-lactam, which is initiated based on data from the first antibiotic susceptibility tests, may fail because of the emergence of resistance mediated by *AmpC* (6). For these reasons, detection of beta-lactamases and monitoring antibiotic susceptibilities are extremely important for the selection of appropriate empirical antibiotic therapy in *P aeruginosa* infections.

The aim of this study is to investigate ESBL, MBL and AmpC beta-lactamase enzymes and *AmpC* gene in *P aeruginosa* strains isolated from clinical specimens sent to the Microbiology Laboratory of Mustafa Kemal University Hospital.

MATERIAL AND METHODS

Bacterial isolates

P aeruginosa strains isolated from clinical specimens sent to the Microbiology Laboratory of Mustafa Kemal University Hospital between June 2011 and September 2012 were included in the study. The strains were stored in tryptic soy broth (Merck, Germany) with 20% glycerin at -70 ˚C.

Identification and determination of antibiotic susceptibilities of the isolates

Isolates were identified as *P aeruginosa* based on colony morphology, odour, Gram staining, production of blue-green pigment on Mueller Hinton agar (MHA), reactions (k/k) on triple sugar iron agar slants and positive oxidase reaction. Identification of the species was confirmed with the Vitek 2 compact system (bioMérieux, France) as required. Antibiotic susceptibilities of these isolates were determined with the Vitek 2 automated system (bioMérieux, France).

ESBL combined disk confirmation test (CDCT)

Bacterial suspensions were adjusted to 0.5 McFarland turbidity and spread evenly in all directions on MHA . Ceftazidime (30 μg) and ceftazidime/clavulanic acid (30/10 μg) disks (Bioanalyse, Turkey) were placed onto the MHA and the plates were incubated for 16–18 hours at 37 ˚C (8). An increase in the zone diameter of ≥ 5 mm for either antimicrobial agent tested in combination with clavulanic acid over that when tested alone was confirmed as ESBL positive strains according to the Clinical and Laboratory Standards Institute (CLSI) criteria (8). *Klebsiella pneumoniae* (*K pneumoniae*) ATCC 700603 and *Escherichia coli* (*E coli*) ATCC 25922 were used as ESBL positive and negative control strains.

Investigation of the presence of MBL

Metallo-beta-lacatamase enzymes were investigated by E-test MBL strips (bioMérieux, France) with IP (4–256 μg/mL) and IP (1–64 μg/mL)-EDTA (IPI). According to the producer's recommendations, minimum inhibitory concentration (MIC)IP/MICIPI ≥ 8 was interpreted as being suggestive of MBL production.

Investigation of the presence of AmpC beta-lactamase

AmpC beta-lactamases were screened by the standard disk induction test (DIT) using imipenem (10 μg) and cefoxitin (30 μg) disks as the inducing substrates and ceftazidime (30 μg), cefotaxime and aztreonam disks (all from Bioanalyse, Turkey) as the reporter substrates. Disks were applied at a distance of 20 mm, and any obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the inducing substrates was interpreted as a positive result for AmpC. The strains that were resistant to extended spectrum cephalosporins, aztreonam and cefoxitin and susceptible to imipenem

were evaluated as constitutive (uninductible-stable derepressed mutant) AmpC beta-lactamase positive.

Detection of *AmpC* genes with the PCR method

DNA extraction from bacteria was performed with DNA extraction kit (Roche, Germany) in accordance with the manufacturer's description.

Amplification of *Amp*C genes

Amplification of *AmpC* genes was performed with polymerase chain reaction (PCR) using the primers of *AmpC* genes that were selected from a research article of Dumas *et al* (9), as shown in Table 1.

Table 1. The primers used in this study

Primer	Oligonucleotide sequence	Product size (bp)
AmpC1	5'-CGGCTCGGTGAGCAAGACCTTC-3'	
AmpC2	5'-AGTCGCGGATCTGTGCCTGGTC-3'	218

The PCR amplification was carried out in a total volume of 25 µL reaction mixture. The reaction mixture consisted of 2.5 µL Taq buffer (10X) [Fermentas, USA], 1.5 µL magnesium chloride ($MgCl_2$) [25 mM; Fermentas, USA], 1 µL dNTP (10 mM) [Fermentas, USA], 0.5 µL (50 pmol) primer, 0.25 µL Taq polimerase [5 U/µL; Fermentas, USA] and 2 µL DNA and brought up to a 25 µL final volume with distilled water. The amplification process was started with an initial denaturation step (94 °C, four minutes). Polymerase chain reaction consisted of 30 cycles of amplification. Amplification consisted of denaturation at 94 °C for one minute, annealing at 57 °C for 45 seconds and DNA chain extension at 72 °C for 45 seconds. And a final extension cycle was performed at 72 °C for 10 minutes. The PCR amplification was performed with Techne Flexigene Thermal Cycler (Techne Flexigene, UK).

The PCR products were analysed in a 2% (w/v) agarose gel in 1X Tris Borate EDTA (TBE) [Wisent, Canada]. Ethidium bromide stained DNA amplicons were visualized using a gel imaging system (Wealtec, Dolphin-View, NV, USA). To determine the expected bp lengths, DNA markers (Fermentas, USA) with defined molecular weights in the range of 50–1031 bp were used. For the presence of *AmpC* gene, 218 bp gene products were evaluated (Fig. 1).

Statistical analysis

Data were analysed using the Statistical Package for Social Sciences version 16.0 (SSPS Inc, Chicago, USA). Comparison for categorical variables was calculated using Chi-squared test. A *p*-value 0.05 was considered statistically significant.

RESULTS

The frequencies of the specimens at different clinics from which *P aeruginosa* isolates were obtained were determined as intensive care units, 30%, Orthopaedics and Traumatology, 14%, Infectious Diseases and Clinical Microbiology, 10%, Plastic and Reconstructive Surgery, 8% and Respiratory Diseases, 8%.

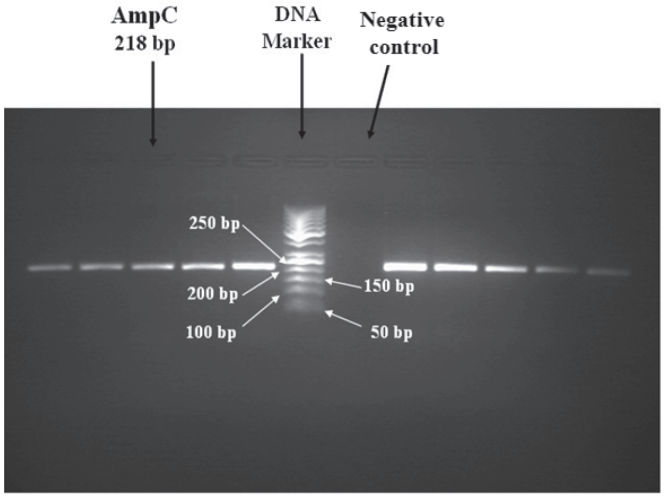

Fig: 1. Polymerase chain reaction products obtained from the gel images of agarose.

Among the strains isolated, 47% were from wounds, 19% from sputum, 18% from urine, 6% from blood, while 6%, 3% and 1% were isolated from tracheal aspirates, bronchoalveolar lavage and pleural fluid, respectively.

The antibiotics to which the strains were the most susceptible in this study were imipenem (89%), amikacin (87%), meropenem (86%), gentamicin (84%) and ciprofloxacin (84%). The highest resistance rate was found to piperacillin/tazobactam (35%), cefepim (26%), levofloxacin (26%), ceftazidime (25%) and piperacillin (21%). The rates of antibiotic resistance and sensitivity of the strains are shown in Fig. 2.

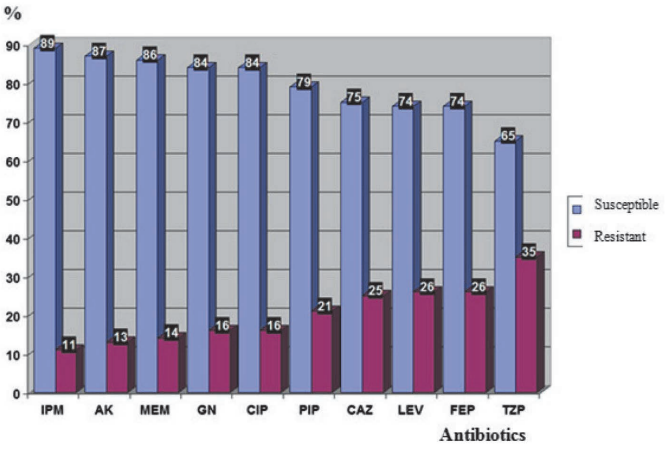

Fig: 2. The rates of antibiotic resistance and sensitivity of the strains.

IPM: imipenem, AK: amikacin, MEM: meropenem, GN: gentamicin, CIP: ciprofloxacin, PIP: piperacillin, CAZ: ceftazidime, LEV: levofloxacin, FEP: cefepim, TZP: piperacillin/tazobactam

According to the MIC_{50} values which were determined with an automated system, the antimicrobials, in the ranges of susceptibilities, were amikacin, gentamicin, ciprofloxacin, levofloxacin, ceftazidime, cefepim, imipenem, meropenem, piperacillin and piperacillin/tazobactam. The antimicrobials except for amikacin, gentamicin, cefepim and imipenem were not in the ranges of susceptibilities according to the MIC_{90}.

Four per cent of strains were determined to be ESBL positive with ceftazidime and ceftazidime/clavulanic acid (CAZ/CZC) CDCT. These strains were MBL negative with E-test method. Three ESBL positive strains were found to be AmpC beta-lactamase negative as none of them was constitutive AmpC beta-lactamase positive. All ESBL positive strains were detected to carry *AmpC* gene with the PCR method.

Extended spectrum beta-lactamase positive strains were found more resistant to amikacin ($p = 0.007$), gentamicin ($p = 0.012$), ciprofloxacin ($p = 0.012$), ceftazidime ($p = 0.003$), cefepime ($p = 0.004$) and piperacillin/tazobactam ($p = 0.013$) than ESBL negative strains. No statistically significant difference between ESBL positive and negative strains was found in resistance to levofloxacin, imipenem, meropenem and piperacillin ($p > 0.05$).

Only one strain (1%) was found to be MBL positive with the E-test method. The MBL positive strain was found to be resistant to amikacin, gentamicin, ciprofloxacin, levofloxacin, ceftazidime, cefepime, imipenem, meropenem, piperacillin, piperacillin/tazobactam and susceptible to aztreonam. This strain was also detected to be ESBL, AmpC beta-lactamase negative and to carry *AmpC* gene with the PCR method.

Seventy-three (73%) of the strains were found to produce AmpC beta-lactamase with DIT and eight of them were evaluated to be constitutive *AmpC* beta-lactamase positive. The strains which were intermediately susceptible to antibiotics were considered as resistant strains so 60 (60%) AmpC beta-lactamase positive strains were determined to be susceptible to ceftazidime and cefepime ($p < 0.05$). Sixty-two (62%) AmpC beta-lactamase positive strains were susceptible to piperacillin/tazobactam, 51 (51%) were found to be susceptible to piperacillin and 70 (70%), 69 (69%) and 60 (60%) AmpC beta-lactamase positive strains were susceptible to imipenem, meropenem and aztreonam, respectively ($p < 0.05$).

Seventy-one (71%) and 70 (70%) strains carrying *AmpC* gene were found to be susceptible to ceftazidime and cefepime, respectively. Among them, 75% were found to be susceptible to piperacillin and 61% susceptible to piperacillin/tazobactam. In addition, the susceptibility rates of strains carrying *AmpC* gene to imipenem, meropenem and aztreonam were found to be 85%, 82% and 72%, respectively.

AmpC gene was detected in 96 (96%) strains with the PCR method. Seventy-three (73%) of these strains were found to produce *AmpC*-beta-lactamase with DIT. The comparison of the presence of *AmpC* gene with PCR and the presence of AmpC beta-lactamase with DIT are shown in Table 2.

Table 2: Comparison of the presence of *AmpC* gene with PCR and the presence of *AmpC* beta-lactamase with disk induction test

Tests and evaluation		**The presence of *AmpC* gene with PCR**			
		Negative n (%)	**Positive n (%)**	**Total**	***p***
The presence of *AmpC* beta-lactamase with disk induction test	Negative	4 (4%)	23 (23%)	27	0.004
	Positive	0 (0%)	73 (73%)	73	

PCR: polymerase chain reaction

DISCUSSION

The production of beta-lactamase is the most important mechanism in the development of antibiotic resistance in *P aeruginosa* strains. These beta-lactamases are *AmpC*, ESBL and carbapenamases (1).

One of the hospital settings where the most resistant bacteria are present is intensive care units. Infections due to resistant bacteria, especially in intensive care units have been increasing over time (10). In this study, *P aeruginosa* strains were the most frequently isolated from the patients in intensive care units. And they were the most frequently isolated from wound, sputum and urine samples.

Carbapenems are beta-lactam antibiotics which are least affected by bacterial resistance. However, acquired carbapenamases, usually found in *Pseudomonas, Acinetobacter* spp and sometimes in *Enterobacteriaceae* members, are reported at increasing rates (11). In this study, the rate of resistance to imipenem and meropenem was found to be 11% and 14%, respectively. In the studies from other countries, the rates were reported to be between 3.4% and 42.3% for imipenem and between 9.1% and 45.5% for meropenem (12–16).

The development of resistance to single antibiotic treatment occurs more frequently in *P aeruginosa* infections. Antipseudomonal antibiotics are therefore generally used in combination with an aminoglycoside. Amikacin is more effective than other members of the group in the infections caused by Gram-negative bacteria including *Pseudomonas,* because it is less affected by aminoglycoside-modifying enzymes (6). In this study, we determined the resistance rates to amikacin and gentamicin to be 13% and 16%, respectively. Also, in other countries, the strains were reported more resistant to gentamicin (12.9–79.7%) than against amikacin [6.5–59.1%] (14–17).

Quinolones can be used alone or in combination treatment. Ciprofloxacin is the most effective in nosocomial infections caused by *P aeruginosa* (6). In this study, the resistance rates to ciprofloxacin and levofloxacin were found to be 16% and 26%, respectively. From other countries, the resistance rates to ciprofloxacin was determined to be between 11.3% and 80.3% (15, 17, 18).

Ceftazidime is prefered in the treatment of *P aeruginosa* infections. Twenty-five per cent of the strains were found to be resistant to ceftazidime in this study. In other studies, the researchers found this rate was between 9.9% and 45.8% (14, 15, 17). The other antibiotic that should be prefered in the treatment of nosocomial infections caused by Gram-negative bacilli is cefepim. This antibiotic is more stable against AmpC beta-lactamases than the third generation cephalosporins and is more effective against *P aeruginosa* strains. The cefepim resistance rate was 26%. In other studies, it was reported to be between 11.2% and 48.9% (12–17, 18).

We found the resistance rate of piperacillin/tazobactam to be 35%. From other countries, the resistance rates of piperacillin and piperacillin/tazobactam were reported to be between 12% and 86.2% and between 9% and 56.8%, respectively (14, 15, 17). Contrary to expectations, in our study, the resistance to piperacillin was found less than that to piperacillin/tazobactam. Because of this, in the induction of AmpC beta-lactamase, the resistance to beta-lactamase inhibitors is considered to be more than that to beta-lactams.

In our study, the resistance rates were found to be less than those in other studies. This situation may be due to moving into a new hospital building, the effective implementation of infection control programmes in hospital and using antibiotics after determination of antibiotic susceptibilities of pathogens.

In the other studies, the rate of ESBL producing in *P aeruginosa* strains was between 14% and 64% (19–21). The ESBL rate (4%) which was determined in this study was found to be the same or less than in some studies. It was thought that the variety of methods used in studies, regional differences in patient profiles and the presence of a high level of AmpC beta-lactamase which was masking the identification of ESBL accounted for this situation.

Metallo-beta-lacatamases are detected as the most dominant enzyme in carbapenemases. Wordwide, MBLs are isolated especially from *P aeruginosa, A baumannii* and, in recent years, from *Enterobacteriaceae* members (22). The usage of beta-lactam antibiotics is seriously restricted in infections caused by MBL-producing bacteria (22). The most important problem in treatment of infections caused by these bacteria is their broad-spectrum resistance profile (22). In our study, one MBL-producing strain was found to be susceptible to aztreonam but resistant to amikacin, gentamicin, ciprofloxacin, levofloxacin, ceftazidime, cefepime, imipenem, meropenem, piperacillin and piperacillin/tazobactam. In other countries, Mendiratta *et al* (23) and Khosravi and Mihani (24) reported MBL rates to be 18.9% and 19.5%, respectively. In all of the studies, including our study, the rates of MBL production vary over a wide range.

AmpC beta-lactamases or cephalosporinases were produced by *P aeruginosa* as well as a majority of *Enterobacteriaceae* members (25). The detection of AmpC beta-lactamases is important for appropriate antibiotic therapy and infection control policies. In other countries, the rate of AmpC beta-lac-tamase production was reported higher than the studies in our country (26, 27). The rate of AmpC beta-lactamase produc-tion detected in this study is in the range of the rates reported from our country. The ratio of stable derepressed mutant (constitutive AmpC beta-lactamase positive) strains was found to be 8% in our study and it is close to that reported by Dunne and Hardin (27). AmpC beta-lactamases are effective benzyl penicillin and cephalosporins (6). Low-level production of the enzyme leads to resistance to amino-penicillins and cephalosporins (6). The resistance to amino-penicillins, beta-lactamase inhibitors and first generation cephalosporins and susceptibility to carboxypenicillins, ureidopenicillins, the third generation cephalosporins, aztreonam, cefepime, cefpirome and carbapenems arise as a result of AmpC beta-lactamase induction (6). In AmpC beta-lactamase-producing strains, resistance to third generation cephalosporins can develop during treatment although initially they are found to be susceptible to third generation cephalosporins with antibiotic susceptibility tests. Although 60% of the strains which were found to produce AmpC beta-lactamase with DIT were determined to be susceptible to ceftazidime, it should be kept in mind that they may develop resistance. In our study, the resistance to piperacillin was found less than that to piperacillin/tazobactam. The frequency of resistance to beta-lactamase inhibitors are seen more than that to beta lactams in the presence of AmpC beta-lactamase induction. The susceptibility to carbapenems occured upon induction of AmpC beta-lactamase. Seventy per cent and sixty-nine per cent of strains in which AmpC beta-lactamase production was found to be positive with DIT were found to be susceptible to imipenem and meropenem, respectively. Also, 60 of 73 strains in which AmpC beta-lactamase production was found positive with DIT were determined to be susceptible to aztreonam.

In this study, 96 (96%) strains were detected to carry *AmpC* gene with the PCR method. In previous years, we found the rate of *AmpC* gene to be 42% in *P aeruginosa* strains isolated from patients in intensive care units of our hospital (28). Therefore, these two studies indicate an increased rate of *AmpC* gene over the years in our hospital. Seventy-three strains which were detected to carry *AmpC* gene with PCR were also detected to produce AmpC beta-lactamase with DIT. There was no production of AmpC beta-lactamase in 23 strains, although they were detected to carry *AmpC* gene. It shows that the gene is not expressed in these strains. *AmpC* gene is now known to be transported by plasmids. Although it is not expressed in the strains, it can be spread rapidly from the strains carrying this gene to the other strains. For these reasons, it is important to check AmpC beta-lactamase, ESBL- and MBL-producing bacteria, for treatment and prevention of the infections caused by them.

Antibiotic resistance is a serious public health problem in our country, as well as all over the world. For this reason, antibiotic resistance mechanisms should be known and the development of resistance should be prevented. Detection of

beta-lactamases and strains carrying genes encoding beta-lactamases will be the guide in the selection of antibiotics, follow-up treatment, preventing infectious diseases and the development of infection control programmes.

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