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Investigation of Antibiotic Resistance Genes and Class 1 Integron in Multi-Drug Resistant *P. aeruginosa* and *E. coli* Strains

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Abstract. *P. aeruginosa* and *E. coli* are resistant to many antibiotics, so the treatment of infections of these pathogens has become difficult. Therefore, searching for the presence of antibiotic resistance genes in clinical isolates is of great importance. The purpose of this study is to investigate the presence of beta-lactam resistance genes and class 1 integron in multi-drug resistant *P. aeruginosa* and *E. coli* clinical strains. Vitek 2 Compact automatization system were used for identification and antibiogram of 2 *P. aeruginosa* and 2 *E. coli* isolates which were isolated from blood, urine and sputum specimens of patients whom were hospitalized in Gümüşhane State Hospital intensive care unit. Total DNA isolation was done by boiling DNA method. PCR were performed using primers of class 1 integron gene cassette, bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{GES}, bla_{CTXM-1}, bla_{CTXM-2}, bla_{OXA-58}, bla_{OXA-23}, bla_{OXA-51}, bla_{OXA-40} and bla_{KPC}. All amplification samples were performed on 1% agarose gel and subsequently visualized under UV light. According to the results of the antibiogram, *P. aeruginosa* isolates showed resistance against all used antibiotics except gentamicin. The *E. coli* isolates were found to be resistant to cephalosporin antibiotics and susceptible to carbapenems. bla_{CTXM-1}-class A beta lactamase genes was found in 3 strains of 4, while no bla_{CTXM-2}, bla_{GES} and bla_{KPC} genes were detected in the strains. The presence of the investigated class B and class D beta lactamase genes was not observed in any sample. The presence of class 1 integron was detected in 3 strains. According to the result of the DNA sequence analysis, it was determined that three integron positive samples had the *dfrA1/AadA5* gene cassettes.

***Keywords*:** Antibiotic resistance, beta-lactamase, integron.

Çoklu-İlaç Dirençli *P. aeruginosa* ve *E.coli* Suşlarında Antibiyotik Direnç Genlerinin ve Sınıf 1 İntegron Gen Kasetlerinin Araştırılması

Özet. *P. aeruginosa* ve *E.coli* izolatlarının birçok antibiyotiğe direnç kazanması nedeniyle bu patojenlerin neden olduğu enfeksiyonların tedavisi zorlaşmıştır. Bundan dolayı klinik izolatlarda antibiyotik direnç genlerinin belirlenmesi büyük önem arz etmektedir. Bu çalışmanın amacı çoklu-ilaç dirençli *P. aeruginosa* ve *E.coli* klinik suşlarında beta laktamaz direnç genlerinin ve sınıf 1 integronlar gen kasetlerinin varlığının araştırılmasıdır. Gümüşhane Devlet Hastanesi yoğun bakım ünitesinde yatan hastaların kan, idrar ve balgam örneklerinden izole edilen 2 *P. aeruginosa* ve 2 *E.coli izolatlarının* tanımlaması ve antibiyogramı Vitek 2 Compact otomatize sistemi ile çalışılmıştır. Total DNA izolasyonu kaynatma DNA metoduyla yapılmıştır. bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{GES}, bla_{CTXM-1}, bla_{CTXM-2}, bla_{OXA-58}, bla_{OXA-23}, bla_{OXA-51}, bla_{OXA-40}, bla_{KPC} ve sınıf 1 integron gen kasetlerine ait primerler kullanılarak PZR'ler gerçekleştirilmiştir. Amplikasyonun gerçekleştiği tüm örnekler %1'lik agaroz jelde yürütülmüştür ve daha sonra UV ışığında görünür hale getirilmiştir. Antibiyogram sonucuna göre *P. aeruginosa* izolatları gentamisin hariç kullanılan tüm antibiyotiklere karşı direnç gösterdiği belirlenmiştir. *E.coli* izolatlarının ise karbapenemlere karşı duyarlı sefalosporin grubu

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antibiyotiklere karşı dirençli olduğu görülmüştür. Sınıf A beta laktamaz genlerinden bla_{CTXM-1} 4 suşun 3'ünde bulunurken, hiçbir suşta bla_{CTXM-2}, bla_{KPC} ve bla_{GES} genleri tespit edilmemiştir. Araştırılan sınıf B ve sınıf D beta laktamaz genlerinin varlığı hiçbir örnekte görülmemiştir. 3 suşta sınıf 1 integronun varlığı tespit edilmiştir. DNA dizi analiz sonucuna göre üç integron pozitif örneğin *dfrA17/AadA5* gen kasetine sahip olduğu belirlenmiştir.

***Anahtar Kelimeler*:** Antibiyotik direnci, beta-laktamaz, integron.

1. INTRODUCTION

One of the major problems of the world is antibiotic resistance in bacteria. The resistance to β-lactam antibiotics is usually provided by β-lactamases [1,2]. The development and rapid diffuse of phytopathogenic strains of *P. aeruginosa* and *E. coli* emerge as a very serious problem in the clinic. There are 4 classes of beta lactamases; Class A (SHV, GES, TEM, CTX-M, KPC); Class B (IMP, VIM, GIM, SPM, and NDM-1); Class C (AmpC) and Class D (oxacillinase). Four group classes beta lactamases were found in *P. aeruginosa* and *E. coli* isolates. Carbapenemases, which cause carbapenem resistance in particular, have been defined in *E. coli* and *P.aeruginosa* isolates in most countries [3, 4].

The development of antibiotic resistance has led to the discovery of many natural moving elements such as transposons and conjugative plasmids. Integrons are carried on plasmids and transposons which cause rapid propagations among species of bacteria. Integrons are genetic elements with region-specific recombination system. These DNA elements provide bacterial integration of antibiotic resistance genes through site-specific recombination [5]. Since its first discovery in clinical isolates, integrons have been identified as a common part of bacterial genomes [5]. Within the last few years, many new resistance gene cassettes emerged. Integrons, which have the ability to transfer many resistance gene cassettes horizontally between species, play an important role in promoting multiple drug resistance among the species Enterobacteriaceae [6-9].

In this study, it was aimed to characterize class 1 integron and beta-lactamase resistance genes in multi-drug resistant *P. aeruginosa* and *E. coli* strains isolated from clinical specimens.

2. MATERIALS AND METHOD

Strains and Antimicrobial Susceptibility Tests

P. aeruginosa and *E. coli* isolates were isolated in Gümüşhane State Hospital. Samples were stored at -20 °C in 20% glycerol. All clinical isolates were identified using Vitek 2 Compact automated system. Ampicillin-sulbactam, piperacillin-tazobactam, ceftazidime, cefotaxime, ceftriaxone, cefepime, doripenem, imipenem, meropenem, amikacin, gentamycin, ciprofloxacin and levofloxacin antibiotics were used for antibiotic susceptibility test and evaluated according to Eucast.

Total DNA Isolation

EMB agar diffusion plates were made from *P. aeruginosa* and *E. coli* isolates in the glycerol stock and left for incubation for 16 hours at 37 °C. After incubation, a single colony was removed from the isolates and seeded in 3 mL of liquid LB medium. 1.5 mL amount of overnight culture was precipitated at 13000 rpm for 1 min. Total DNA isolation was obtained by boiling and the pellet was dissolved in 500 µL of deionized water. The cells were lysed by heating at 95 °C for 10 min. Supernatants were used in the PCR reactions.

Polymerase Chain Reaction for Class 1 Integron

The presence of class 1 integron in *P. aeruginosa* and *E. coli* strains was investigated by the PCR method. The primers used for the detection of the integron are shown in Table 1. PCR was prepared as such: 1.5 units of DNA polymerase I (GoTaq, Promega), 5 µL of DNA, 10 µL of 5X DNA polymerase buffer (Promega), 3 µL of 1.5 mM $MgCl_2$, 2.5 µL of 4 mM each dNTP and 1.5 µL of each a primer stock (25 pmol / µL) and a final volume of 50 µL with sterile deionized water.

Amplification for integron was performed at 94 °C for 3 min (initial denaturation), 45 s at 94 °C, 1 min at 55 ° C, 3 min at 72 °C (34 cycles) and final synthesis for 5 min at 72 °C. PCR products were run on agarose gel electrophoresis and visualized in UV. Positive samples were sent to Sentegen Company for DNA sequence analysis.

Determination of Beta-lactamases by PCR

The oligonucleotides used in PCR are shown in Table 1. PCR was prepared as such: 1.5 units of DNA polymerase I (GoTaq, Promega), 5 µL of DNA, 10 µL of 5X DNA polymerase buffer (Promega), 3 µL of 1.5 mM $MgCl_2$, 2.5 µL of 4 mM each dNTP and 1.5 µL of each a primer stock (25 pmol / µL) and a final volume of 50 µL with sterile deionized water. The T_m's used for PCR amplification are given in Table 1. PCR products were run on agarose gel electrophoresis and visualized in UV.

Table 1. Specific primers and Tm used in the study.

Primer	5'-3'	Amplicon size (bp)	T_m	References
OXA-51	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353	52°C	21
OXA-23	F: GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCCAT	501		
OXA-24	F: GGTTAGTTGGCCCCCTTAAA R: AGTTGAGCGAAAAGGGGATT	246		
OXA-58	F: AAGTATTGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	599		
GES	F:ATGCGCTTCATTCACGCAC R:CTATTTGTCCGTGCTCAGGA	863	56°C	22
IMP	F:CATGGTTTGGTGGTTCTTGT R:ATAATTTGGCGGACTTTGGC	488	56°C	23
VIM	F:ATTGGTCTATTTGACCGCGTC R:TGCTACTCAACGACTGAGCG	780	58°C	
CTX-M1 group	F:GCGTGATACCACTTCACCTC R:TGAAGTAAGTGACCAGAATC	260	50°C	24
CTX-M2 group	F:TGATACCACCACGCCGCTC R:TATTGCATCAGAAACCGTGGG	341	50°C	
NDM	F: GAGATTGCCGAGCGACTTG R: CGAATGTCTGGCAGCACACTT	457	54°C	25
Class 1 integron	5'CS:5′-GGCATCCAAGCAGCAAG-3′ 3'CS:5′-AAGCAGACTT ACCTGA-3′	-	55°C	25
KPC	F: ATGTCACTGTATCGCCGTCT R: TTTTCAGAGCCTTACTGCCC	538	58°C	25

Evaluation of Sequence Results

The results of the samples sent to Sentegene for DNA base sequence analysis were evaluated using Expasy (http://web.expasy.org/translate/) and Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) programs.

3. RESULTS

2 *P.aeruginosa* and 2 *E. coli* strains that were included in the study were taken from Gümüşhane State Hospital. One of *P.aeruginosa* isolates was isolated from sputum and the other was isolated from the urine sample. *E. coli* isolates were isolated from blood and urine specimens. The antibiotic resistance profiles of the strains are shown in Table 2. Accordingly, *P.aeruginosa* (Pa1 and Pa2) isolates were found to be resistant to beta-lactamase inhibitors (piperacillin-tazobactam), aminoglycosides (amikacin), carbapenems (doripenem, imipenem, meropenem), cephalosporins (ceftazidime and cefepime) and quinolones (ciprofloxacin and levofloxacin). The antibiotic susceptibility profiles of *E. coli* (Ec1 and Ec2) strains were resistant to ampicillin-sulbactam, cephalosporins (ceftazidime, cefotaxime, ceftriaxone) and quinolones (ciprofloxacin, levofloxacin), while they were found to be sensitive aminoglycosides (amikacin, gentamycin)

and carbapenems (doripenem, imipenem, meropenem). According to antibiotic susceptibility profiles, 4 isolates were observed to be multidrug resistant. PCR analysis was performed for the detection of genes encoding class A, class B and class D beta lactamase. The class B carbapenemase (*bla*$_{IMP}$, *bla*$_{NDM}$ and *bla*$_{VIM}$) and class D beta lactamase (*bla*$_{OXA-23}$, *bla*$_{OXA-40}$, *bla*$_{OXA-58}$ and *bla*$_{OXA-51}$) were not detected in any isolates. Only the CTXM-1-group gene (class A beta lactamase) was detected in Pa2, Ec1 and Ec2 isolates.

PCR analysis showed that the presence of class 1 integron was found in 3 out of 4 strains (Pa2, Ec1, and Ec2) and the size of the integrons was approximately 1800 bp. According to DNA sequence analysis, three samples were found to have the *dFrA17/AadA5* gene cassette sequence.

Table 2. Antibiotic Resistance Profile for Clinical Samples.

Antibiotics Group	Antibiotics	Pa1	Pa2	Ec1	Ec2
β-lactam / β-lactamase inhibitor	Ampicillin-sulbactam	-	-	R	R
	Piperacillin-Tazobactam	R	R	S	S
Cephalosporin	Ceftazidime	R	R	R	R
	Cefotaxime	-	-	R	R
	Ceftriaxone	-	-	R	R
	Cefepime	R	R	-	-
Carbapenem	Doripenem	R	R	S	S
	İmipenem	R	R	S	S
	Meropenem	R	R	S	S
Aminoglycosides	Amikacin	R	R	S	S
	Gentamicin	S	S	S	S
Quinolone	Ciprofloxacin	R	R	R	R
	Levofloxacin	R	R	R	R

4. DISCUSSION AND CONCLUSION

Strains resistant to at least one of the antibiotics included in three or more antibiotic groups are defined as multidrug resistance (MDR) [10]. Antibiotic groups and antibiotics included in these groups have been reported by Magiorakos et al. [10]. By using the definition of MDR, it was determined that 4 isolates were multidrug resistant.

Resistance to all antibiotic group are seen in *P. aeruginosa* and *E. coli* strains. The quick diffusion of antibiotic-resistant genes among bacteria has become a serious problem worldwide [11]. Many investigations have shown that the resistance genes are transferred by the integrons. Most resistance determinants are found in gene cassettes [12]. Class 1 integrons are also shown worldwide in *P. aeruginosa* and *E. coli*. In these studies, the frequencies of class integrons were found 41.5% (44/106) in Brazil, 60% in England, 59% in France and 52% in Taiwan [11,13]. In Turkey, in a multicenter study of clinical *P. aeruginosa* isolates, the frequency of class 1 integrons was found to be 4.8% [14]. Resistance genes that cause aminoglycosides and β-lactams are frequently found on integrons in Pseudomonas and Enterobacteriaceae members.

The most prevalent mechanism of aminoglycoside resistance are Aad and Aac families [13,14]. In the isolates of our study, the most common resistance is the aadA family (aadA5) and the dfrA family (*dfrA17*). The *dfrA17/aadA5* gene cassette was detected in *P. aeruginosa* and *E. coli* strains. This gene cassette gives resistance to streptomycin, spectinomycin and trimethoprim. This gene cassette has also been reported in different bacterial species and in other regions [15]. The *dfrA17/aadA5* gene cassette can be spread by plasmids. For this reason, clinically resistant isolates can be explained by the widespread presence of increased integrons [15].

Beginning in 1995, CTX-M type ESBLs have emerged all over the world [16,17]. There are 5 groups of CTX-M type ESBL; CTX-M-1-2-8-9-25. Among these enzymes, the CTX-M 15 from the

CTX-M group is the most common one found in the world [18,19]. A study of multi-drug resistant *K. pneumoniae* isolates showed that CTX-M-type ESBL is quite common in Turkey. Another multicenter study in Turkey showed that CTX-M 71% is the most common enzyme among *K. pneumoniae* and *E. coli* isolates[19]. In this study, CTX-M-type ESBL was detected in 1 *P. aeruginosa* isolate and 2 *E.coli* strains.

There are many causes of carbapenem resistance in *P. aeruginosa*. Plasmid or integrons, an increase in the expression of efflux systems, an increase in porin expression and an increase in chromosomal cephalosporinase activity have been identified as factors contributing to carbapenem resistance [20]. In this work, carbapenemases such as bla_{VIM}, bla_{NDM}, bla_{IMP} were not detected in carbapenem resistant *P. aeruginosa* strains (Pa1 and Pa2). It is thought that carbapenem resistance in Pa1 and Pa2 isolates may be caused by one or more of the other mechanisms mentioned above.

It is important to know the presence of class 1 integrons and the distribution of these isolates in hospitalized patients in multidrug resistant *P. aeruginosa* and *E. coli* strains. As a result, integrons play a crucial role in spreading antibiotic resistance among clinical isolates. For this reason, it is important to monitor the presence and distribution of antibiotic resistance genes by techniques such as PCR and sequence analysis.

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