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First report of qnr and *bla*$_{\text{VIM-4-like}}$ producing clinical *Alcaligenes faecalis* isolated in Türkiye

Türkiye'den izole edilen qnr ve bla$_{\text{VIM-4-like}}$ *üreten klinik Alcaligenes faecalis'in ilk raporu*

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Abstract

The study set out to look at the clinical strain of *A. faecalis*'s antibiotic susceptibility pattern. Both biochemical and molecular approaches were used to identify *A.faecalis*. The VITEK compact system was used to analyze the strain's antibiotic susceptibility profile. The use of PCR was evaluated to determine the presence of resistance genes. It was also determined whether resistance determinants could be transferred. It was determined that *A. feacalis* showed resistance to imipenem, cefepime, ceftazidime, gentamicin, amikacin, netilmicin, tobramicin, ciprofloxacin, and levofloxacin. The *bla*$_{\text{VIM-4-like}}$ gene was present in this strain, according to the results of the PCR and DNA sequencing studies. In contrast to VIM-4, this *bla*$_{\text{VIM-4-like}}$ gene showed one amino acid change (Ala265Val).Filter-mating experiments showed that the *bla*$_{\text{VIM-4-like}}$ gene cannot be transferred from *A. faecalis* to *E. coli*. In this study, This research, demonstrated the presence of the *bla*$_{\text{VIM-4-like}}$ gene for the first time in *A. faecalis* from Turkey.

Keywords*: A. faecalis*, qnrB, VIM-4

Öz

Çalışmanın amacı, A. faecalis klinik suşunun antibiyotik duyarlılık paterninin araştırmaktır. A. faecalis hem biyokimyasal hem de moleküler yöntemlerle tanımlanmıştır. VITEK kompakt sistemi, suşun antibiyotik duyarlılık profilini analiz etmek için kullanıldı. Direnç genlerinin belirlenmesi, PCR yöntemi kullanılarak değerlendirildi. Ayrıca, direnç belirleyicilerinin aktarılabilirliği araştırıldı. A. feacalis'in imipenem, sefepim, seftazidim, gentamisin, amikasin, netilmisin, tobramisin, siprofloksasin ve levofloksasine direnç gösterdiği belirlendi. PCR ve DNA dizi analizleri, bu suşun bla$_{\text{VIM-4}}$ *benzeri gene sahip olduğunu ortaya çıkardı. Bu bla*$_{\text{VIM-4-like}}$ *gen, VIM-4'e kıyasla bir amino asit ikamesi (Ala265Val) sergiledi. Filtre eşleştirme deneyleri, bla*$_{\text{VIM-4-like}}$ *genin A. faecalis'ten E. coli'ye aktarılamayacağını gösterdi. Bu çalışmada, Türkiye'den A. faecalis'te ilk kez bla*$_{\text{VIM-4-like}}$ *genin varlığı ortaya konmuştur.*

Anahtar kelimeler*: A. faecalis, qnrB, VIM-4*

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1. Introduction

Gram-negative, aerobic, rod-shaped *Alcaligenes faecalis* bacteria are found in soil and water. They are oxidase, catalase, and citrate positive. (Pereira et al., 2000; Khajuria et al., 2013). *A. faecalis* can cause dangerous opportunistic infections and is also present in the microbiome of humans. (Pereira et al., 2000). This organism often causes nosocomial infections including sporadic endocarditis, meningitis, chronic otitis, urinary tract infections, infantile gastroenteritis, pyelonephritis, bacteremia, peritonitis, endophthalmitis, nosocomial pseudobacteremia, and abscesses (Tena et al., 2015; Kavuncuoglu et al., 2010; Khokhar et al., 2002; Ashwath & Katner, 2005) and is usually seen in immunocompromised hosts (Tena et al.,2015).
A. faecalis is typically not susceptible to aminoglycosides, chloramphenicol, or tetracyclines; however, it is susceptible to trimethoprim/sulfamethoxazole (TMP-SMX). Moreover, it is usually susceptible to β-lactams such as ureidopenicillin, ticarcillin-clavulanic acid, cephalosporins, and carbapenems (Wisplinghoff, 2017). Aminoglycoside resistance may occur in strains by different mechanisms. One of which is the inactivation or modification of aminoglycosides (Doi et al., 2016). Aminoglycoside phosphotransferase encoding genes; *strA, strB, aphA6* (Mantengoli & Rossolini, 2005), aminoglycoside nucleotidyltransferase encoding gene; *ANT (2'')-Ia* (Al Laham et al., 2017), aminoglycoside acetyltransferase encoding genes; *aacA4* (Al Laham et al., 2017), *aac(3)-II* (Hidalgo del Río, 2014), *aac(6')-I* (Dubois et al., 2006), *Aac-6'-Ib-cr* (De Paiva, 2015), *AAC(3)-II-b* (Hidalgo del Río, 2014), and aminoglycoside adenyltransferase encoding genes; *aadA2* (Adelowo & Fagade, 2012), *aadB* (Al Laham et al., 2017), *aadA11* (Agersø & Sandvang, 2005), *aadA1* (Barlow et al., 2008) were found in *A. faecalis*. Five class integrons have been identified to date and these genetic structures play major role in spreading antibiotic resistance genes (Cury et al., 2016). Class I and Class II integrons were found in *A. faecalis*. aadB-bla_{VIM-2} -dfrA34-aacA4-dfrB5 (Al Laham et al., 2017), aadA2-sul-1B-qacEΔ1-F (Adelowo & Fagade, 2012), aadA11-dfrA1 (Agersø & Sandvang, 2005), and aadA1 (Barlow et al., 2008) gene cassette arrays with integrase I gene were identified in this bacteria. β-lactamases are enzymes found in various bacterial species that can inactivate chemical compounds like penicillins. β-Lactamases are composed of Class A, B, C and D (Bush, 2018). β-lactamases production is one of the most important causes of multidrug resistance among bacteria. (Bush, 2010). Class A β-lactamase PER-1 (Mantengoli & Rossolini, 2005), TEM-21 (Dubois et al., 2006), SHV (Adesoji & Ogunjobi, 2016), CTX-M1 (Zeynudin et al., 2018) and class B metallo βlactamase VIM-4 (Al Laham et al., 2017), VIM-2 (Al Laham et al., 2017), VIM-6 (Khajuria et al., 2013), NDM-1 (Wang et al., 2013), IMP-1 coding genes have been reported in *A faecalis*. Efflux of the drug are cellular formations that provide resistance to antibiotics. More than 40 genes (tet-genes) encoding tetracycline resistance have been characterized to date, and most of them encode membrane-associated efflux proteins (Møller et al., 2016). The second most common tetracycline resistance flow pump, *tetA* (Møller et al., 2016) was reported in *A. faecalis* isolates. Fluoroquinolones are potent, antibiotics for the cure of severe or resistant infections. However, resistance to fluoroquinolones has been developing in recent years and causes problems in clinical settings. Plasmid-mediated quinolone resistance (PMQR) genes cause resistance to fluoroquinolones. The first report of this gene is the presence of the qnrA gene on the plasmid in *K. pneumoniae*. (Redgrave et al., 2014). Fluoroquinolones resistant *A. faecalis* isolates have been reported in Angola (Filipe et al., 2017).
In this paper, it was focused to determine the antibiotic resistance pattern and analyze the transferability of the resistance in *A. feacalis* isolated from the urine sample of a patient in Trabzon Fatih State Hospital in Türkiye.

2. Material and method

2.1. Bacterial strain and antimicrobial susceptibility test

From urine cultures taken in 2017 at Trabzon Fatih State Hospital, an *A. faecalis* clinical strain was identified. Utilizing the VITEK system 2 Compact automated system (biomeriux, France), the strain's biochemistry was identified, and tests for antibiotic susceptibility were run.
The results were verified using 16S rDNA (Primers 27F: AGAGTTTGATCMTGGCTCAG, 1492R GGYTACCTTGTTACGACTT used in 16S rDNA). Antibiotic susceptibility testing was conducted using cefepime, aztreonam, piperacillin, ceftazidime, imipenem, meropenem, amikacin, gentamicin, netilmicin, tobramycin, ciprofloxacin, and colistin. The results were assessed in accordance with the EUCAST 2017 guidelines (EUCAST, 2017).

2.2. Total DNA isolation

The isolate was inoculated into 3 ml of antibiotic-free Luria-Bertani (LB) medium and incubated overnight at 37°C for 16 hours. The sample's DNA was extracted from the bacterial culture using the boiling procedure. A 1 minute, 10,000 rpm centrifugation was performed on 1 ml of the culture. After discarding the supernatant and washing the pellet once in 1 mL of sterile water, the pellet was given another 1 mL of sterile water. The suspension was centrifuged for 10 minutes at a speed of 13000 rpm after boiling it for 10 minutes at 100 °C. The supernatant was stored for use in PCR after centrifugation (Çıçek et al., 2013).

2.3. Detection of antibiotic resistance genes and integrons by PCR

bla$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-48}$, *bla*$_{OXA-58}$, *bla*$_{VIM}$, *bla*$_{VEB}$, *bla*$_{GIM}$, *bla*$_{PER}$, *bla*$_{IMP}$, *bla*$_{GES}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTXM-1}$, *bla*$_{CTXM-2}$, *bla*$_{NDM}$, *bla*$_{SIM}$, qnrA, qnrB, qnrS, aac6-Ib and integrons primers used in the PCR reaction (Table 1). Reaction buffer (5 µL), $MgCI_2$ (3 µL 25 mm), dNTP (1 µL 4 mm), primers 10mM (1 µL), genomic DNA (5 µL), and GO Taq polymerase (1 U) were prepared to a final volume of 50 microliters. PCR products were run on an ethidium bromide-containing 1% agarose gel and then visualized using UV light and the outcomes were assessed in light of the positive controls' molecular weights, which were discovered to include antibiotic resistance genes in earlier investigations.

Table 1. Primers Used in Polymerase Chain Reaction

Primer	5'-3'	References
bla$_{OXA-48}$	F: TTGGTGGCATCGATTATCGG R: AGCACTTCTTTTGTGATGGC	Iraz et al., 2014
bla$_{OXA-23}$	F: GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCCAT	Woodford et al., 2006
bla$_{OXA-24}$	F: GGTTAGTTGGCCCCCTTAAA R: AGTTGAGCGAAAAGGGGATT	Woodford et al.,2006
bla$_{OXA-58}$	F: AAGTATTGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	Woodford et al.,2006
bla$_{GES}$	F: ATGCGCTTCATTCACGCAC R: CTATTTGTCCGTGCTCAGGA	Moubareck et al.,2009
bla$_{VEB}$	F:ATTTCCCGATGCAAAGCGT R: TTATTCCGGAAGTCCCTGT	Moubareck et al., 2009
bla$_{PER-2}$	F: ATGAATGTCATCACAAAATG R: TCAATCCGGACTCACT	Celenza et al.,2006
bla$_{IMP}$	F: CATGGTTTGGTGGTTCTTGT R: ATAATTTGGCGGACTTTGGC	Jeon et al.,2005
bla$_{VIM}$	F: ATTGGTCTATTTGACCGCGTC R: TGCTACTCAACGACTGAGCG	Jeon et al.,2005
bla$_{NDM-1}$	F: GAGATTGCCGAGCGACTTG R: CGAATGTCTGGCAGCACACTT	Cicek et al.,2014
qnrS	F: ACGACATTCGTCAACTGCAA R: TCTAAACCGTCGAGTTCGGCG	Vasilaki et al.,2008; Cattoir et al.,2007
qnrA	F: AGAGGATTTCTCACGCCAGG R: CCAGGCACAGATCTTGAC	Vasilaki et al.,2008
qnrB	F: GGMATHGAAATTCGCCACTG R: TTTGCYGYYCGCCAGTCGAA	Cattoir et al.,2007
bla$_{TEM}$	F: AGTATTCAACATTTYCGTGT R: TAATCAGTGAGGCACCTATCTC	Copur Cicek et al.,2013a
bla$_{SHV}$	F: ATGCGTTATATTCGCCTGTG R: TTAGCGTTGCCAGTGCTC	Copur Cicek et al.,2013a
bla$_{CTX-M1}$	F: GCGTGATACCACTTCACCTC R: TGAAGTAAGTGACCAGAATC	Copur Cicek et al.,2013a
bla$_{CTX-M2}$	F: TGATACCACCACGCCGCTC R: TATTGCATCAGAAACCGTGGG	Copur Cicek et al.,2013a
bla$_{GIM}$	F: TCGACACACCTTGGTCTGAA R: AACTTCCAACTTTGCCATGC	Ellington vd., 2007; Woodford 2010
bla$_{SIM}$	F: TACAAGGGATTCGGCATCG R: TAATGGCCTGTTCCCATGTG	Ellington et al.,2007; Woodford 2010
*intI*1	F: ACATGTGATGGCGACGCACGA R: ATTTCTGTCCTGGCTGGCGA	Copur Cicek et al.,2013b
*intI*2	F: CACGGATATGCGACAAAAAGGT R: GTAGCAAACGAGTGACGAAATG	Copur Cicek et al.,2013b

2.4. Cloning of bla$_{VIM-type}$ Metallo-Beta Lactamase Gene

A cloning experiment was performed to detect genes encoding *bla*$_{VIM-like}$ in our strain. Ligation experiment was performed according to the guide in the kit (pGEM®-T Easy Vector Systems, A1360). Ligation Product was transferred to *E. coli DH5a* strain and DNA isolation was performed from positive plasmids obtained after transformation. All plasmid DNAs were sent to Sentegen (Ankara) for DNA sequence analysis. Sequence results were evaluated using the CLUSTAL W and BLAST bioinformatics programs. The DNA sequence was uploaded to GenBank.

2.5. Transferability of antibiotic resistance by conjugation

The resistance transfer test was carried out using conjugation experiments. (Rice et al., 1990). Equal amounts of the donor *(A. fecalis*) and receiver (*E. coli* K-12 strain J53-2) bacteria were planted in antibiotic-free Luria-Bertani media before being cultured at 37°C for 20 hours. After incubation, transconjugants were identified using Eosin-Methylene Blue (EMB) agar.

3. Results and discussion

A. faecalis was isolated from urine culture. Both molecular and biochemical techniques were used to identify isolated A. faecalis. The isolate underwent a VITEK test for antibiotic susceptibility, and the outcomes were assessed in accordance with EUCAST 2017 (EUCAST 2017). The isolate was positive for resistance to ceftazidime, cefepime, imipenem, amikacin, gentamicin, netilmicin, tobramicin, ciprofloxacin, and levofloxacin in the antibiotic susceptibility test. The strain was found to be moderately sensitive to aztreonam and meropenem. Additionally, piperacillin and colistin were effective against *A. faecalis*. (Table 2). PCR was used to check for βlactamase genes (*bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-48}$, *bla*$_{OXA-58}$, *bla*$_{VIM}$, *bla*$_{VEB}$, *bla*$_{GIM}$, *bla*$_{PER}$, *bla*$_{IMP}$, *bla*$_{GES}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTXM-1}$, *bla*$_{CTXM-2}$, *bla*$_{NDM}$, *bla*$_{SIM}$), quinolone resistance genes (qnrA, qnrB and qnrS) and Class I and II integrons. The strain was found to carry the qnrB (Figure 1). The isolate was also class I integron positive.

Table 2. Antibiotic susceptibility profile of *A. faecalis*

Antibiotics	MIC (µg/mL)	R/S/I
Piperacillin	16	S
Ceftazidime	>=64	R
Cefepime	>=32	R
Aztreonam	16	I
Imipenem	>=16	R
Meropenem	8	I
Amikacin	>=64	R
Gentamicin	8	R
Netilmicin	>=32	R

R/S/I values were evaluated according to EUCAST.
(R:Resistant, S: sensitive, I: moderately sensitive)

Among the βlactamase resistance genes investigated, the strain carried only VIM-type metallo-β lactamase (Figure 1). After being cloned into the pGEMT, the *bla*$_{VIM}$ gene was forwarded for sequencing analysis. The sequence result showed that this gene was 99% similar *bla*$_{VIM-4}$. A single amino acid was found to be different between VIM-like and VIM-4. (A265V). Therefore, this VIM-like was found to be the new VIM variant and it is named to VIM-4-like. The nucleotide sequence of the VIM-4-like was uploaded to the Genebank (accession number: MN792829).

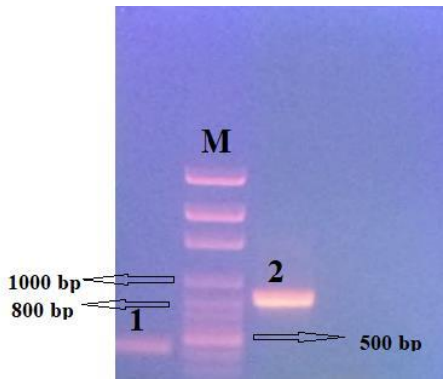

Figure 1. PCR gel image of samples (1:qnrB, M: Marker, 2 :VIM)

Conjugation experiments were performed between *A. faecalis* and *E.coli K-12* strain J53-2 and no transconjugant was obtained.

The gram-negative, oxidase-positive, rod-shaped *A. faecalis* is classified as an environmental bacterium and rarely causes clinically important infections in humans (Kahveci et al., 2011). The most frequent reason for technical failure in peritoneal dialysis (PD) is likely peritonitis, a major side effect of PD. 16% of individuals with PD die as a result of this infection (Szeto et al., 2011). *A. faecalis* peritonitis was reported in two cases of PD from Türkiye. These patients recovered under antimicrobial therapy alone, and catheter removal was not necessary. (Kavuncuoglu et al., 2010; Kahveci et al., 2011).

A. faecalis that was resistant to carbapenems, polymyxins, and even tigecycline was isolated in the blood culture of a 60-year-old female patient with a history of diabetes mellitus and hypertension in the ICU of a hospital in Bangladesh (Hasan et al., 2019). Skin and soft tissue infections from the University of Guadalajara Hospital (Spain) were examined and *A. faecalis* was isolated from five patients. As a result of this study, it was reported that in particular, post-operative patients and individuals with vascular disorders should be aware of this organism's potential pathogenicity (Tena et al., 2015).
In this study, *A. faecalis* that was resistant to ceftazidime, cefepime, imipenem, amikacin, gentamicin, netilmicin, tobramicin, ciprofloxacin, and levofloxacin was isolated from a flaxide tetraplagy patient in Türkiye.

Fluoroquinolone (ciprofloxacin, levofloxacin), cephalosporin (cefepime, ceftazidime, cefotaxime), aminoglycoside (gentamisin, tobramycin), carbapenem (imipenem, meropenem), penicillin (ampicillin, piperacillin), β-lactam/β-lactamase inhibitor combination (amoxicillin-clavulanate, piperacillin-tazobactam), aztreonam, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline resistance has been observed in different *A. faecalis* isolates from Angola, Bangladesh, Spain (Filipe et al., 2017; Hasan et al., 2019; Tena et al., 2015).

Because quinolones are frequently used to treat urinary tract infections, there has been an increase in quinolone resistance. Many distinct enterobacterial species, including *K. pneumoniae*, Enterobacter spp., *E. coli*, and *Salmonella enterica* isolates, have been identified to carry the genes qnrA, qnrB, and qnrS. (Poirel et al., 2012). *qnrB* genes are more common than other qnr genes. *A. faecalis* clinic isolate had qnrB found in this paper. The resistance of the strain to fluoroquinolones such as ciprofloxacin and levofloxacin may be associated with the presence of *qnrB*.

Five isolates carrying VIM from Gaza were found to be intermediate resistant or resistant to imipenem, but susceptible to meropenem except one isolate (Al Laham et al., 2017). Similar to this result, we determined that the isolate we studied was resistant to imipenem and intermediate resistant to meropenem. This is likely to be the result of the effect of the VIM type carbapenemase on carbapenem susceptibility profile. Because VIM type carbanemases have higher imipenem hydrolysis activities than those of meropenem (Al Laham et al., 2017).
By facilitating horizontal gene transfer between Gram-negative bacteria, integrons, one of the mobile element types referred to as natural gene capture systems in bacteria, are crucial in the spread of antibiotic

resistance. (Copur Çıçek et al., 2013). Using primers tailored to conserved areas, the presence of class I and class II integrons in the *A. faecalis* isolate was examined. It was discovered that the isolate only possessed a class I integron. This non-fermentative uncommon Gram negative bacterium had class I and class II integrons with various gene cassette arrays, and the origin of these integrons was choromosamal or plasmid. (Al Laham et al., 2017; Adelowo & Fagade, 2012; Agersø & Sandvang, 2005; Barlow et al., 2008). The presence of aac6-Ib gene in *A. faecalis* isolate which was resistant to the tested aminoglycoside antibiotics was investigated by PCR and the gene was not found. Another allele of aminoglycoside acetyltransferase or one of the other aminoglycoside modifying enzymes may cause aminoglycoside resistance in this strain. Only the subclass B1 metallo beta lactamase encoding bla_{VIM} was detected among the investigated genes (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, bla_{OXA-58}, bla_{VIM}, bla_{VEB}, bla_{GIM}, bla_{PER}, bla_{IMP}, bla_{GES}, bla_{TEM}, bla_{SHV}, bla_{CTXM-1}, bla_{CTXM-2}, bla_{NDM}, bla_{SIM}, qnrA, qnrB, qnrS) in *A. faecalis* isolate. The sequence result of bla_{VIM} showed that this sequence was 99% differ from VIM-4 with one amino acid substitution (A265V).
This amino acid exchange of VIM-type metallo β-lactamases has not previously been observed in *A. faecalis*. According to our knowledge, VIM-4-like with A265V amino acid substitution was found for the first time in *A. faecalis* in Türkiye. In previous studies, new allele of VIM type metallo βlactamases (VIM-5, VIM-38) have been reported in *P. aeruginosa* in Türkiye (Iraz et al., 2014). There was an amino acid difference between VIM-5 and VIM-38, which was A265V. Results of a study that characterized the kinetic and biochemical properties of VIM-5 and VIM-38 revealed that they had remarkably similar catalytic activities against β-lactam substrates (Makena et al., 2015).

In a study, the conjugation assay was performed using the clinical strain *A. faecalis* Af1930 (with bla_{TEM-21} gene) as donor and azide resistant *Escherichia coli* C600, rifampicin and nalidixic acid resistant *E. coli* K12 or rifampicin resistant mutant *P. aeruginosa* ATCC 27853 strains as recipient. As a result of this study, ESBL producing transconjugant could not be obtained. In addition, the plasmid was isolated from Af1930 could not be transferred to *E. coli DH5α* (Dubois et al., 2006). In another study, it was found that tetA and int1 genes from *A.faecalis* can be transferred together to *Psedomonas putida* by conjugation. *A. faecalis* with conjugative plasmid was isolated from manured soil and pigsty environment. This suggests that *A. faecalis* contribute to the horizontal transfer of resistance genes. (Agersø & Sandvang, 2005). As a result of our conjugation experiment using *A. faecalis* isolate harboring the VIM-4-like gene (as a donor) and *E.coli* strain (as a recipient), we could not obtain any transconjugate. These two results suggest that the $bla_{VIM-4-like}$ gene and other identified genes (qnrB, class I integron gene cassette) are of chromosomal origin. In contrast to our results, VIM-4 was found in integron originated from plasmid (Al Laham et al., 2017).

4. Conclusions

In *A. faecalis* isolate derived from clinical sample, antibiotic resistance was identified. Although not regularly, infections brought on by *A. faecalis* isolates resistant to antibiotics have been documented from various parts of the world. *A. faecalis* has been shown to have β-lactamases such PER-1, TEM-21, SHV, CTX-M1, VIM-4, VIM-2, VIM-6, NDM-1, and IMP-1 that lead to β-lactam resistance. Our study is the first to document the existence of *an A. faecalis* isolate harboring VIM in Türkiye.

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Author contribution

Azer Özad Düzgün, Ayşegül Saral Sarıyer and Esma Akyıldız carried out the experiment and wrote the manuscript with support from Fatih Şaban BERİŞ. Tuba Köse and Mikail Arslan collected the strains and performed antibiograms.

Declaration of ethical code

We hereby undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the

aforementioned penalties can be carried out under the heading "Actions Contrary to Scientific Research and Publication Ethics".
Ethics Committee decision date; 08.11.2017 and number: 23618724

Conflicts of interest

The authors declare no conflicts of interest.

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