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NEXT-GENERATION SEQUENCING OF PLASMID CARRYING *bla*$_{\text{OXA-48}}$ IN *KLEBSIELLA PNEUMONIAE* FROM TURKEY

AZER ÖZAD DÜZGÜN[1,2] and AYŞEGÜL SARAL[3]*

[1]Department of Genetics and Bioengineering, Faculty of Engineering and Natural Sciences, Gümüşhane University, Gümüşhane, Turkey
[2]Medicinal Plants, Traditional Medicine Practice and Research Center, Gümüşhane University, Gümüşhane, Turkey
[3]Department of Nutrition and Dietetics, Faculty of Health Sciences, Artvin Coruh University, Artvin, Turkey

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A carbapenem-resistant *Klebsiella pneumoniae* strain was isolated in Turkey in 2012 and *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ genes were observed in this strain. The aim of this study was to investigate transferability of plasmid bearing *bla*$_{\text{OXA-48}}$ in *K. pneumoniae* and to use whole-genome sequencing in order to understand the genetic context of plasmid. *K. pneumoniae* strain was used as donor in conjugation experiments. Antibiotic susceptibility profile of selected transconjugant was determined. Plasmid was isolated from transconjugant colony and was named as pKPT. Complete sequencing of the pKPT was conducted using a next-generation sequencing. Annotation of the contigs was performed using the Geneious R9, followed by finding open reading frames (ORFs) with selected web-based tools. BLAST analysis was performed at the NCBI BLAST server to determine genes showing more than 90% similarity with these ORFs. Results of antibiotic susceptibility test showed that transconjugant colony was resistant to ampicillin/sulbactam, piperacillin, and piperacillin/tazobactam. The pKPT plasmid had a length of 45,217 bp and an average G + C content of 49%. Blast analysis revealed that pKPT was included in the IncL/M incompatibility group. The pKPT was found to contain *bla*$_{\text{OXA-48}}$ within Tn*1999.2* transposon without any other antibiotic resistance gene.

Keywords: *Klebsiella pneumoniae*, plasmid, *bla*$_{\text{OXA-48}}$, next-generation sequencing, conjugation

Introduction

Conjugative systems in Gram-negative bacteria encourage the horizontal transfer of resistance determinants between species, genera, and kingdoms subject

*Corresponding author; E-mail: asaral@artvin.edu.tr

to their conjugative properties and efficiency of conjugation [1, 2]. Plasmids are one of the most important problems in successful dissemination of antibiotic resistance especially in the spread of carbapenemases, including VIM, IMP, NDM, KPC and OXA β-lactamases (carbapenem-hydrolyzing enzymes) [2]. The presence of plasmid-mediated antimicrobial resistance in *Enterobacteriaceae* is high [2].

Plasmid-encoded class D β-lactamases are OXA-23-like, OXA-40-like, OXA-58-like, OXA-51-like, OXA-143-like, and OXA-48-like [3]. OXA-48 was first isolated from a patient in Turkey in 2001 and it has been described in *Klebsiella pneumoniae* [4]. Although OXA-48 is able to catalyze the hydrolysis of penicillins with high level, it has low-level hydrolytic activity against carbapenems [5, 6]. After the discovery of OXA-48, it was observed worldwide and OXA-48 has been isolated from *Enterobacter cloacae, K. pneumoniae, Escherichia coli, Shewanella xiamenensis, Citrobacter freundii, Serratia marcescens, Providencia rettgeri, Klebsiella oxytoca, Enterobacter sakazakii*, and *Acinetobacter baumannii* [5]. Altogether 11 variants of OXA-48-like were recorded to date (OXA-48, OXA-162, OXA-163, OXA-181, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-370, and OXA-405) [5].

Previous studies showed that plasmids carrying the $bla_{\text{OXA-48}}$ gene obtained from different isolates and different countries have similar properties. They are very similar in size and have only $bla_{\text{OXA-48}}$ as resistance marker with some exceptions [7]. A 70-kb plasmid harboring $bla_{\text{OXA-48}}$ was found in *K. pneumoniae, P. rettgeri, E. cloacae, C. freundii,* and *E. coli* isolated in Turkey [8]. Another 70-kb plasmid with $bla_{\text{OXA-48}}$ gene was found in *K. pneumoniae* isolated in Spain [9]. Unlike the cases above, 160-kb plasmid with $bla_{\text{OXA-48}}$ gene was isolated in *K. pneumoniae* strain from France [10]. Plasmid incompatibility groups of $bla_{\text{OXA-48}}$-positive plasmids were found as IncL/M, IncA/C, and IncF in the previous studies. Plasmids with $bla_{\text{OXA-48}}$ isolated from 107 enterobacterial isolates recovered from European and North African countries and incompatibility groups of 92.5% of these plasmids were found as IncL/M type [11]. *K. pneumoniae* RJ119 isolated in China and 61,748-bp IncL/M conjugative plasmid with $bla_{\text{OXA-48}}$ was found in this isolate [12]. Plasmids belong to IncA/C with $bla_{\text{OXA-48}}$ and $bla_{\text{CMY-4}}$ genes found in *K. pneumoniae* was isolated in Tunisia [13]. The $bla_{\text{OXA-48}}$ gene is located on transposon Tn*1999*. Both ends of Tn*1999* contain IS*1999* elements. Different Tn*1999* isoforms (Tn*1999.2*, Tn*1999.3*, and Tn*1999.4*) have been found in genetic environment of the $bla_{\text{OXA-48}}$ [14]. In a study completed in United Kingdom, $bla_{\text{OXA-48}}$ genes were found in 13 isolates of *K. pneumoniae*, 10 *E. coli*, and 2 *E. cloacae*. These $bla_{\text{OXA-48}}$ genes were located within either Tn*1999* or Tn*1999.2* transposons without other resistance genes [15]. In another study conducted in China, $bla_{\text{OXA-48}}$ genes were found located within Tn*1999.2* transposons in the 69,069-bp plasmid [14].

Materials and Methods

Bacterial strain

Previously, *K. pneumoniae* strains were collected during the years of 2012–2013 and were screened for $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ genes by polymerase chain reaction (PCR) [16]. $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes carrying *K. pneumoniae* strains were observed in this study. One of these strains was used as a donor in conjugation experiment.

Transferability of antibiotic resistance

Transferability of the antibiotic resistance was carried out using a defined protocol. *E. coli* K-12 strain J53-2 (F met pro Rif^{r}) was used as a recipient [17]. Donor (*K. pneumoniae* strain) and recipient cells were inoculated in the same volume (1:1) and incubated at 37 °C for 24 h. The transconjugants were selected on eosin–methylene blue agar (Oxoid, UK). Minimum inhibitory concentration (MIC) values against transconjugant were determined and the following antibiotics were tested: ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, netilmicin, ciprofloxacin, levofloxacin, tigecycline, colistin, and trimethoprim–sulfamethoxazole.

Detection of bla_{OXA-48} *and* bla_{NDM-1} *genes in transconjugants by PCRs*

Plasmid DNA isolation was performed from the transconjugants by Plasmid Purification Kits (Promega, Madison, WI, USA). Transconjugants were screened for $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes by PCR. The primers used to amplify $bla_{\text{NDM-1}}$ (F: TGGAATTGCCCAATATTATGC; R: TCAGCGCAGCTTGTCGGCCATGC) and $bla_{\text{OXA-48}}$ (A: TTGGTGGCATCGATTATCGG; B: GAGCACTTCTTTTGTGATGGC) genes. A single reaction mixture containing 5 µl of genomic DNA, 20 pM of each primer, 10 µl of reaction buffer, 3 µl of 25mM $MgCl_2$, 200 µM dNTPs, and 1.5 U Go Taq Flexi Polymerase (Promega, Madison, WI, USA) with a final volume of 50 µl was employed. All PCR results were analyzed on 1% agarose containing 0.5 µg/ml ethidium bromide, and subsequently visualized under UV light.

Next-generation sequencing of plasmid

Next-generation sequencing of plasmid was carried out commercially by Illumina MiseqTM (Thermo Fisher Scientific, Waltham, MA, USA). Quality

control of raw read sets, which can be significant in genome assembly, was determined with FastQC (https://www.bioinformatics.babraham.ac.uk) [18]. After the evaluation of quality control analysis, extraction of low-quality sequences and trimmimg of sequences were completed through Geneious R9 (https://www.geneious.com). Quality analysis of trimmed 63.918 reads were made with FastQC. The De novo assembly was made by Geneious R9 as well. After genome assembly, basic local alignment search tool (BLAST) analysis was performed to determine the reference sequence that showed maximum similarity and coverage with contigs. For circularizing of plasmid genome, contig1 (45,217 bp) and reference genome (GenBank: NZ_CP018461) were used in run of map to read algorithm of Geneious R9. All ORFs larger than 75 bp found in the consensus sequence obtained by the map to read algorithm were observed using the ORF Finder program (https://www.ncbi.nlm.nih.gov/orffinder/). Genes belonging to the plasmid genome were identified by PlasMapper program (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC441548/?tool=pubmed/). The results were transferred to the GenBank database and the genes that showed more than 90% similarity with characterized sequences of the genes were determined.

Results

Conjugation experiments were performed to determine whether the existing antibiotic resistance genes $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ in *K. pneumoniae* were transferable. According to conjugation experiment result, one transconjugant was obtained and MIC values of some antibiotics against transconjugant were determined. The transconjugant showed high-level resistance to ampicillin/sulbactam (MICs >32 mg/L), piperacillin (MICs ≥128 mg/L), and piperacillin/tazobactam (MICs ≥128 mg/L). In addition, MIC values of imipenem and meropenem (2 mg/L) for transconjugant are higher than the values for *E. coli* K-12 strain J53-2 (Table I). Plasmid DNA isolation was performed from the transconjugant and plasmid was screened for $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes by PCR. The $bla_{\text{OXA-48}}$ gene was found in the plasmid but $bla_{\text{NDM-1}}$ gene was not detected.

Read sets obtained by subtracting the low-quality sequences and combining the sequences was used in genome construction. Using the gene algorithm of Geneious R9, 9,692 contigs were obtained. Those above 1,000 bp and high quality are used in subsequent analyses. As a result of contigs BLAST analysis, it was determined that contig1 (45,217 bp) showed maximum identity and coverage with *K. pneumoniae* strain Kp_Goe_39795 plasmid pKp_Goe_795-2, complete sequence (GenBank: NZ_CP018461). NZ_CP018461 was used as reference genome for circularizing contig1 (45,217 bp) and getting consensus sequence.

Table I. MIC values of transconjugant

	EUCAST MIC breakpoints of *Enterobacterales* (mg/L) [19]		*E. coli* K-12 strain J53-2	Transconjugant
Antibiotics	R	S	MIC (mg/L)/antibiotic susceptibility	
Ampicillin–sulbactam	>8	≤8	4/S	≥32/R
Piperacillin	>16	≤8	≤4/S	≥128/R
Piperacillin–tazobactam	>16	≤8	–	≥128/R
Ceftazidime	>4	≤1	≤1/S	≤1/S
Cefepime	>4	≤1	≤1/S	≤1/S
Imipenem	>8	≤2	≤0.25/S	2/S
Meropenem	>8	≤2	≤0.25/S	2/S
Amikacin	>16	≤8	≤2/S	≤2/S
Gentamicin	>4	≤2	≤1/S	≤1/S
Netilmicin	>4	≤2	≤1/S	≤1/S
Ciprofloxacin	>0.5	≤0.25	≤0.25/S	≤0.25/S
Levofloxacin	>1	≤0.5	≤0.12/S	≤0.12/S
Tigecycline	>2	≤1	≤0.5/S	≤0.5/S
Colistin	>2	≤2	≤0.5/S	≤0.5/S
Trimethoprim–sulfamethoxazole	>4	≤2	≤0.20/S	≤0.20/S

Note: MIC: minimum inhibitory concentration; EUCAST: European Committee on Antimicrobial Susceptibility Testing; R: resistant; S: sensitive.

ORFs were found in consensus sequence with ORF Finder program (https://www.ncbi.nlm.nih.gov/orffinder/). The genes in the plasmid genome that showed similarity over 90% to the genes in the GenBank and the functions of these genes can be seen in Table II. GC content of plasmid was determined as about 49%.

Nucleotide sequence accession number

The complete nucleotide sequence of pKPT was deposited under GenBank accession number MK088079.

Discussion

K. pneumoniae was isolated from tracheal aspirates in 2012 from Turkey. Resistance to ampicillin, ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, ceftazidime, cefoperazone/sulbactam, colistin, amikacin, netilmicin, ciprofloxacin, levofloxacin, tetracycline, imipenem, and meropenem were found in *K. pneumoniae*. The antibiotic resistance-encoding genes of this strain were detected as bla_{NDM-1}, bla_{OXA-48}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, bla_{TEM}, and bla_{SHV},

Table II. Functions of genes found in pKPT

Gene product	Gene position	Roles of gene products in bacteria	References
Phospholipase D family protein	397–807	Virulence factor	[20]
Sugar ABC transporter substrate-binding protein	1,345–1,590	Exporting of a wide variety of cell-surface glycoconjugates	[21]
KorC protein	3,882–4,148	Transcriptional repressor	[12]
Cobalamin biosynthesis protein	5,856–6,035	Synthesizing cobalamin	[22]
Antirestriction protein	9,421–9,861	Facilitieting transformation	[23]
Single-stranded DNA-binding protein	10,637–11,071	Binding to and protecting susceptible ssDNA	[24]
Mobilization protein C, B, A	12,609–15,532	Pilus assembly	[25]
Conjugal transfer protein Tra H, I, J, K, C, L, M, N, O, P, Q, R, U, W, X, Y	13,249–34,964	Conjugal transfer	[26]
ExcA-like protein	34,966–35,619	Part of entry exclusion system of conjugative plasmids	[9]
Replication protein C, A	35,700–37,170	Replication initiation	[27]
Conjugal transfer protein Trb C, B, A, N	38,655–43,298	Conjugal transfer	[26]
Transfer inhibition protein Tir	43,986–44,456	Repression of conjugative plasmid transfer	[28]
IS*1999* transposase	44,435–45,643	Transposition	[29]
LysR family transcriptional regulator	45,950–46,861	Signal transduction regulatory	[30]
OXA-48	47,163–47,960	Carbapenem hydrolysis	[29]
IS1 transposase	48,179–48,451	Transposition	[31]
IS*1999* transposase	48,870–50,078	Transposition	[29]
Transfer inhibition protein Tir	50,127–50,318	Repression of conjugative plasmid transfer	[28]
Protein antitoxin PemI	50,410–50,667	Part of addiction mechanisms	[32]
Cognate toxin PemK	50,669–51,001	Part of addiction mechanisms	[32]
DNA polymerase vs. subunit UmuD	51,094–51,528	DNA reparation	[33]
DNA polymerase vs. subunit UmuC	51,477–52,781	DNA reparation	[33]
Restriction endonuclease	58,867–59,454	Cleaving DNA	[34]
Plasmid partition protein	61,181–62,155	Plasmid partitioning	[35]

previously [16]. In this study, the transferability of resistance genes from *K. pneumoniae* to *E. coli* K-12 strain J53-2 by conjugation experiment was examined. Transconjugant was observed as a result of conjugation experiment. Susceptibility testing of the transconjugant showed that it was resistant to ampicillin/sulbactam, piperacillin, and piperacillin/tazobactam. The MIC of meropenem and imipenem for the transconjugant was sevenfold higher than that for the *E. coli* K-12 strain J53-2 but transconjugant still remained susceptible to imipenem and meropenem (Table I). The results of the antibiotic susceptibility test appear to reflect the substrate hydrolysis profile of OXA-48 (hydrolysis of penicillins with high level and hydrolysis of carbapenems with low level) [5, 6]. The imipenem and meropenem resistance may be traceless to carriage of $bla_{\text{NDM-1}}$ in clinical isolate. Plasmid isolation was performed from transconjugant and isolated plasmid named pKPT. PCR amplification revealed that only $bla_{\text{OXA-48}}$ was found in pKPT. Absence of $bla_{\text{NDM-1}}$ in pKPT may depend on the possibilities that $bla_{\text{NDM-1}}$ may be chromosomal or carried with another plasmid.

Plasmid harboring $bla_{\text{OXA-48}}$ (pKPT) was fully sequenced using Illumina MiseqTM commercially. pKPT was 45,217 bp in size with a G + C content of 49%. BLAST analysis showed that pKPT has high similarity with pKp_Goe_795-2 (CP018461.1; 99% identity and 99% coverage), pOXA-48a (JN626286.1; 99% identity and 97% coverage), pEC745_OXA48 (CP015075.2; 99% identity and 99% coverage), and pKPoxa-48N2 (KC757417.2; 99% identity and 99% coverage).

In a recent study, $bla_{\text{OXA-48}}$ gene-harboring plasmid was extracted from the *E. coli* transconjugant recovered from the *K. pneumoniae* 11978 clinical isolate from Turkey [7]. Plasmid named pOXA-48a was sequenced with Illumina genome analyzer. Size and G + C content of pOXA-48a were found to be 61,881 bp and 51%, respectively. It was determined that pOXA-48a shares 97% nucleotide identity with pCTX-M3. pCTX-M3 and pHK-NDM were used as reference genomes to annotate pOXA-48a [7]. According to our results, genetic environment of $bla_{\text{OXA-48}}$ genes located in pOXA-48a and pKPT was very similar with the exception IS*1999* truncated by *IS1R* element in pKPT (Figure 1). Transposons with the presence of an *IS1R* element truncating the IS*1999* insertion sequence upstream of $bla_{\text{OXA-48}}$ are called Tn*1999.2* [36]. *Tir* gene was truncated by Tn*1999* and Tn*1999.2* in both plasmids (pOXA-48a and pKPT). IS*1999* was shown to provide −35 and −10 promoter sequences for the expression of the $bla_{\text{OXA-48}}$ gene in *K. pneumoniae* 11978. IS*1999* truncated by *IS1* element provides stronger promotor than IS*1999* [29]. It can be speculated that $bla_{\text{OXA-48}}$ gene expression in *K. pneumoniae* in our strain is higher than *K. pneumoniae* 11978 (Figure 2).

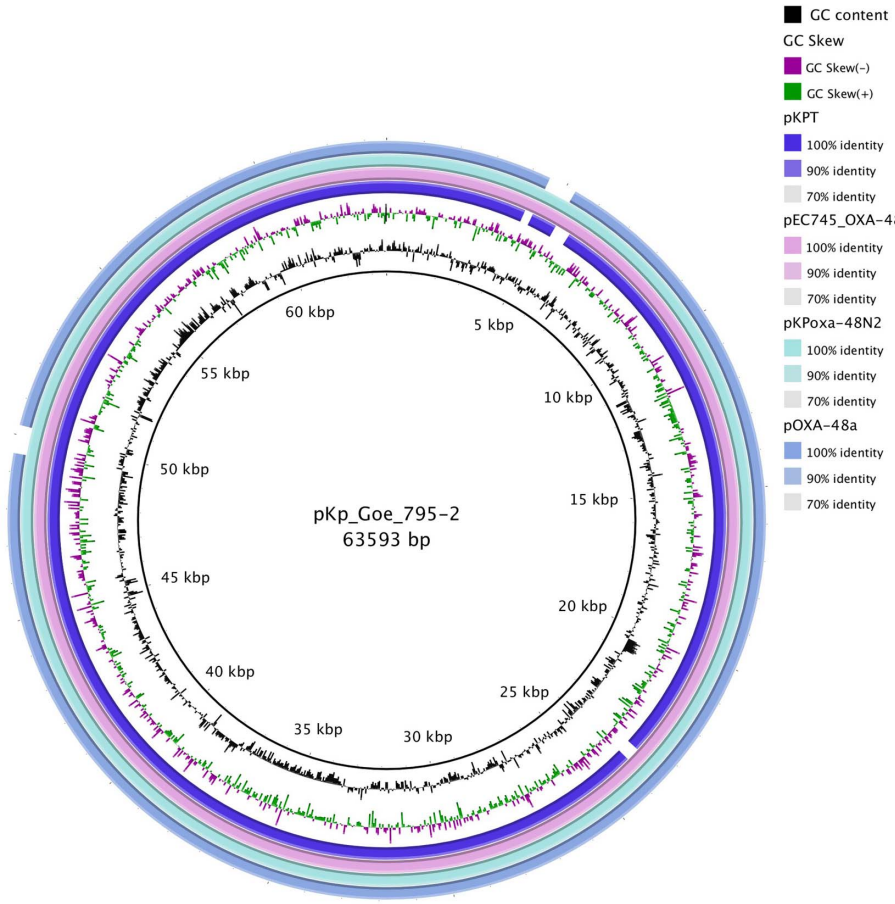

Figure 1. Blast Ring Image Generator comparison of pKp_Goe_795-2 (CP018461.1), pOXA-48a (JN626286.1), pEC745_OXA48 (CP015075.2), pKPoxa-48N2 (KC757417.2), and pKPT (MK088079). Each ring corresponds to a plasmid (indicated at the right of the figure together with the color code). pKp_Goe_795-2 was used as a reference sequence [37]

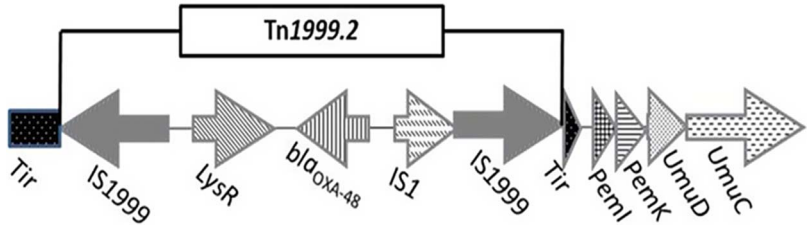

Figure 2. Schematic representation of genetic environment of *bla*$_{\text{OXA-48}}$

The *tra* and *trb* genes encoding proteins involved in conjugal transfer enable the spread of plasmids and resistance determinants [26]. The transfer region of pKPT is composed of 15 *tra* genes and 4 *trb* genes (*traH, traI, traJ, traK, traL, traM, traN, traO, traP, traQ, traR, traU, traW, traX, traY, trbC, trbA, trbB,* and *trbC*; Table II). In addition, *mobCAB* genes encoding proteins have role in pilus assembly and plasmid conjugative apparatus [25]. The *mobCAB* genes were observed in pKPT with BLAST analysis.

The *repA*, *parA*, and *traU* genes are used as molecular markers for the detection of all IncL/M plasmids [9]. Nucleotide sequence of *repA* of pKPT shares high similarity with *repA* of pKp_Goe_795-2 (CP018461.1; 98% identity and 100% coverage), pOXA-48a (JN626286.1; 98% identity and 100% coverage), pEC745_OXA48 (CP015075.2; 98% identity and 100% coverage), and pKPoxa-48N2 (KC757417.2; 98% identity and 100% coverage). On the other hand, *traU* and *parA* of pKPT have same nucleotide sequence with *traU* and *parA* of pKp_Goe_795-2 (CP018461.1; 100% identity and 100% coverage), pOXA-48a (JN626286.1; 100% identity and 100% coverage), pEC745_OXA48 (CP015075.2; 100% identity and 100% coverage), and pKPoxa-48N2 (KC757417.2; 100% identity and 100% coverage). Based on these observations, it is concluded that plasmid incompatibility group of pKPT is IncL/M type.

It was found that epidemic pOXA-48 plasmids share very similar backbone with other IncL/M plasmids except for *traX, traY, excA*, and *traX* nucleotide sequences used in classification of plasmids [9]. BLAST analysis showed that the *traX, traY*, and *excA* of pKPT have same nucleotide sequence with *traX, traY*, and *excA* of pKp_Goe_795-2 (CP018461.1; 100% identity and 100% coverage), pOXA-48a (JN626286.1; 100% identity and 100% coverage), pEC745_OXA48 (CP015075.2; 100% identity and 100% coverage), and pKPoxa-48N2 (KC757417.2; 100% identity and 100% coverage). It can be elucidated that all these plasmids are in same phylogenetic group.

Large, low-copy, self-transmitting resistance plasmids in the *Enterobacteriaceae* have role in dissemination of antibiotic resistance [32]. During outbreaks caused by multiresistant *Enterobacteriaceae*, the plasmid-coded resistance determinants can be transferred to different species. Next-generation sequencing is a molecular technique to identify resistance plasmids [38]. In this study, pKPT harboring bla_{OXA-48} was characterized. pKPT showed identical structures within bla_{OXA-48}-harboring plasmids, such as pKp_Goe_795-2 (CP018461.1), pOXA-48a (JN626286.1), pEC745_OXA48 (CP015075.2), and pKPoxa-48N2 (KC757417.2). Similar to pOXA-48a, pKPT may be responsible for the spread of bla_{OXA-48} among *Enterobacteriaceae* in Turkey [10]. To the best of our knowledge, plasmid harboring bla_{OXA-48} with the Tn*1999.2* transposon detected in *K. pneumoniae* from Turkey was completely sequenced for the first time.

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Conflict of Interest

The authors declare no conflict of interest.

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