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RESEARCH ARTICLE

Characteristics in the whole-genome sequence of *Klebsiella pneumoniae* ST147 from Turkey

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ABSTRACT

The study aimed to analyze antibiotic resistance determinants in a carbapenem-resistant *Klebsiella pneumoniae* by whole-genome sequencing (WGS). *K. pneumoniae* was isolated from a urine sample and it was characterized by 16S rDNA sequencing in Turkey. This strain was named as *Kpn* Rize-53-TR. Antimicrobial susceptibility testing was performed for seventeen antibiotics by VITEK-2 and the result was confirmed by MIC. The whole genome of isolate was sequenced by Illumina and was analysed by bioinformatic tools for MLST, replicon types, and antimicrobial resistance genes. The whole genome data was submitted to NCBI. The isolate was found to be resistant to all tested β-lactam antibiotics and the highest MIC values were found for piperacillin, piperacillin/tazobactam (≥128). No resistance to colistin and moderate susceptibility to amikacin and tetracycline was observed. The isolate carried 12 resistance genes belonging to 10 resistance classes; *ere*(A), *fosA, oqxB, cml*A1, *aac(a)-IIa,* $bla_{\text{KPC-2}}$, $bla_{\text{TEM-1A}}$, $bla_{\text{SHV-67}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1-2-9}}$. Mutations were detected in *gyrA* (83Y) and *parC* (80I) genes. Clonal subtype of the isolate was ST147, and it had wzi420 and wzc38 alleles. Its serotype was O3/O3a. The $bla_{\text{KPC-2}}$ was firstly found in both ST147 clonal group in Turkey and in serotype O3/O3a in the world. By plasmid replicon typing, five plasmids IncFII(K), Col(BS512), IncR, IncFIA(HI1) and IncFIB(pQil) were determined in *Kpn* Rize-53-TR and $bla_{\text{KPC-2}}$ was located on IncFII(K) plasmid. The presence of $bla_{\text{KPC-2}}$ on the plasmid with other resistance genes accelerates its own spread together with other resistance genes.

KEYWORDS

carbapenemase, *Klebsiella pneumoniae*, ST147, serotype O3/O3a, KPC-2, IncFII(K), whole-genome sequencing

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INTRODUCTION

Klebsiella pneumoniae, one of the most common opportunistic pathogens, is naturally present in the human intestinal tract. Its diversity and epidemiology have dramatically changed by the time. The expression of extended-spectrum β-lactamases (ESBLs) is the main mechanism of resistance of *K. pneumoniae* strains and confer resistance to nearly all β-lactams except carbapenems. Carbapenem resistance is a hot topic among *K. pneumoniae* isolates in both developed and developing countries and it can be caused by loss or alterations

of outer membrane porins (OMPs) often combined with β-lactamase production or acquisition of carbapenemases [1–3]. Clinically, three major types of acquired carbapenemases are recognized in *K. pneumoniae* and in other *Enterobacteriaceae* according to the Ambler classification, including the molecular class A *K. pneumoniae* carbapenemase (KPC)-type, the class B metallo-β-lactamases (MBLs; mainly IMP-, VIM-, and NDM-type enzymes) and the class D OXA-48-like enzymes [1–2]. Among these, the most commonly seen are KPC, NDM and OXA-48.

KPC is capable of hydrolyzing a variety of β-lactams, including penicillins, cephalosporins, carbapenems, and aztreonam [4]. KPC is encoded by more the 90 alleles of the bla_{KPC} gene (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/KPC) and it is responsible for the most common transmissible carbapenem resistance mechanisms worldwide. The dissemination of bla_{KPC} has been historically associated with distinct *K. pneumoniae* lineages, a particular plasmid family and a composite transposon. In the United Kingdom, spread of bla_{KPC} appears to have been supported by highly fluid, modular exchange of larger genetic segments among plasmid populations dominated by IncFIB, IncFII, and IncR replicons [5, 6]. Besides, aminoglycoside modifying enzymes, regulators of pump clearance systems, pore defects, AmpC, ESBL and fluoroquinolone-resistance determinants are also important for the development of resistance [7].

The whole genome sequence analysis is an important approach to reveal information such as all resistance genes that bacteria may have, virulence genes, information from the clonal group and to look at the genome in detail. We performed the first whole genome analysis of bla_{KPC-2} carbapenemase-coding *K. pneumoniae* from Turkey.

MATERIALS AND METHODS

Bacterial strain and antimicrobial susceptibility testing

Clinical isolate of *K. pneumoniae* was isolated from a urine sample and was identified by standard microbiological procedures and VITEK-2 (bioMerieux, Marcy-l'Etoile, France) at Recep Tayyip Erdoğan University Hospital in Turkey. It was also confirmed by 16S rDNA sequencing and named as *Kpn* Rize-53-TR. Susceptibility testing was performed by using the following antibiotics: piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, netilmicin, tobramycin, ciprofloxacin, levofloxacin, tetracycline, tigecycline, colistin and trimethoprim/sulfamethoxazole and was confirmed by E-test (bioMerieux) in accordance with the CLSI guidelines [8].

Whole-Genome Sequencing (WGS)

Kpn Rize-53-TR strain showed resistant phenotype to antibiotics from different groups and especially to all β-lactam antibiotics, therefore it was decided to determine the whole genome sequence in order to reveal the resistance gene profile. Bacterial sample was sent to Macrogen NGS Service in Europe for the preparation and library constraction of isolate. The library was sequenced by an Illumina SBS technology and converted into raw data for analysis. The sequence reads were de novo assembled validated using mapping strategy and BUSCO analysis. After whole genome or draft genome was assembled, the location of protein-coding sequences, tRNA genes, rRNA genes, and tmRNA genes were identified. Then their functions were annotated. Prokka was used to predict the location while BLAST was used to get to know function and identification of assembled sequences against nucleotide and protein sequences database.

Analysis of genome

Replicase genes of the plasmids were classified using the PlasmidFinder-2.0 online tool. Multilocus sequence type (MLST) was identified using whole-genome sequences in the MLST database by bioinformatics tools available from the Center for Genomic Epidemiology (https://genomicepidemiology.org/). Antimicrobial resistance encoding genes were identified using ResFinder and used a cutoff of 80% similarity.

RESULTS

Resistance profiles

K. pneumoniae was found resistant to all tested β-lactam antibiotics. The highest MIC values were found for piperacillin, piperacillin/tazobactam (≥128). The MICs for carbapenems (imipenem and meropenem) were determined and these showed more than 16 µg ml^{-1} value (Table 1). When the results were evaluated for non-β-lactam antibiotics, moderate susceptibility to amikacin and tetracycline was observed, while no resistance to colistin was observed and it was used as the only effective antibiotic.

WGS. The whole genome was 5,577,550 nucleotides in length and it was submitted to GenBank under the accession JABMCK000000000. It had the coding sequence for a total of 5307 genes, 78 tRNAs, 9 rRNAs.

Plasmid types. A total of 5 plasmids were detected in the genome of *Kpn* Rize-53-TR. These plasmids are IncFII(K), Col(BS512), IncR, IncFIA(HI1) and IncFIB(pQil). While Col(BS512), IncR and IncFIB(pQil) were 100% similar in replicon typing; IncFII(K) and IncFIA(HI1) had nucleotide changes and they were found to be 97.97% and 98.45% similar, respectively. The plasmids identified in this study and plasmids in database that have the maximum similarity are shown in Table 2. Of the resistance genes, only bla_{KPC-2} was detected on IncFII(K). None of the other resistance genes were carried on the plasmid. The isolate belonged to ST147 clone and there are 96 isolates in databases at the same MLST.

Table 1. Antibiotic resistance profiles and MIC values

Antimicrobials	Evaluation	MICs	Antibiotic group
β-lactam antibiotic			
Piperacillin	R	≥=128	Ureidopenicillin
Piperacillin/ Tazobactam	R	≥=128	Ureidopenicillin
Ceftazidime	R	≥=64	Third-Generation Cephalosporin
Cefepime	R	≥=32	Fourth-generation Cephalosporin
Aztreonam	R	≥=64	Monobactam
Imipenem	R	≥=16	Carbapenem
Meropenem	R	≥=16	Carbapenem
Non-β-lactam antibiotic			
Amikacin	I	16	Aminoglycoside
Gentamicin	R	≥=16	Broad spectrum aminoglycoside
Netilmicin	R	≥=32	Aminoglycoside
Tobramycin	R	≥=16	Aminoglycoside
Ciprofloxacin	R	≥=4	Fluoroquinolone
Levofloxacin	R	≥=8	Fluoroquinolone
Tetracycline	I	8	Tetracyclin
Tigecycline	R	≥=8	Glycylcycline
Colistin	S	≤=0.5	Cyclic polypeptide
Trimethoprim/ Sulfamethoxazole	R	80	Sulfonamide

Antibiotic resistance genes. A total of 12 resistance genes were identified. These are *ere*(A) (macrolide resistance), *fos*A (fosfomycin resistance), *oqxB* (quinolone resistance), *cml*A1 (phenicol resistance), *aac(a)-IIa* (aminoglycoside resistance), $bla_{\text{KPC-2}}$ (carbapenem resistance), $bla_{\text{TEM-1A}}$, $bla_{\text{SHV-67}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{OXA-9}}$ (β-lactam resistance). Two virulence factors were determined as *traT* and *iutA*. The *traT* appeared on the IncFII(K) plasmid along with $bla_{\text{KPC-2}}$. We found alteration in *gyrA* gene (83Y) and in *parC* gene (80I) together with *oqxAB* and these determinants confer resistance to quinolones or to fluoroquinolones. The isolate harbored numerous MDR efflux pump genes such as CmeA, CmeB, MATE, MFS, MacA, MarcB, MarA, OML, RND, AcrB and AcrAB. Its serotype was determined such as O3/O3a.

Discussion

K. pneumoniae has been classified as an ESKAPE organism (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) and it is a major source of antibiotic resistance with its high prevalence [12]. The increasing resistance rates of *K. pneumoniae* and the occurrence of ESBL producing *Klebsiella pneumonia* worldwide are serious epidemic topics in many countries [13, 14]. In addition, carbapenemases that confer resistance to all classes of antibiotics makes treatment of infections caused by *K. pneumoniae* much more difficult. Identifying all of the resistance genes a pathogen has, is crucial to understanding the resistance profile and the reasons behind it. There is a complete agreement between the resistance genes determined by WGS analysis and the antibiotic profile.

Quinolones hinder bacterial DNA replication by blocking the topoisomerases and they have been used in clinical practice since the 1960s. Its extensive use led to the development of bacterial quinolone resistance mechanisms and *K. pneumoniae* resistome brought together all the resistance mechanisms known for quinolone resistance [15, 16]. The *oqxB* was detected in *Kpn* Rize-53-TR, as well as point mutations in *gyrA* and *parC* genes responsible for quinolone resistance. Changes in *K. pneumoniae* cell permeability have been reported to be involved in quinolone resistance and the pump, *oqxAB*, is one of them [17]. But, the first and major resistance mechanism is chromosomal mutation in the quinolone binding targets of DNA gyrase (*gyrA-gyrB* subunits) and topoisomerase IV (*parC-parE* subunits) [14]. Both these determinants are responsible for the observed quinolone resistance in *Kpn* Rize-53-TR. Moreover, no tetracyclin resistance and colistin resistance genes were found in the genome, which explained the sensitivity to these antibiotics. In a previous study that we conducted with 37 carbapenem-resistant strains, colistin was the most effective antibiotic [18].

KPC is an important enzyme for carbapenem resistance and it hydrolyzes cephalosporins, monobactams, and carbapenems (imipenem and ertapenem). The mortality for patients infected with KPC-producing *K. pneumoniae* is high as a result of the limited antibiotic options such as colistin, tigecycline, or aminoglycosides. The first KPC has emerged in the United States in 1996 [19] and since then, bla_{KPC} carrying strains have been described in all continents. The $bla_{\text{KPC-2}}$ and $bla_{\text{KPC-3}}$ genes are already the most common variants in hospital outbreaks in many countries [20].

There may be a strong relationship between antibiotic resistance genes and sequence types (STs) clonal groups. It is important to reveal this relationship in terms of country and continents. This will affect the direction and success of the therapies to be used. The dissemination and population structure of carbapenem resistance *K. pneumoniae* is also

Table 2. The plasmids with the most similarity found in the databases

Plasmids	Identity	Accession number	Plasmide sizes (bp)	References	Reference bacterium
Col(BS512)	100	NC010656	2089	*Shigella boydii* CDC 3083-94	Direct Submission
IncFIA(HI1)	98.45	AF250878	180,461	*Salmonella typhi* R27	[9]
IncFIB(pQil)	100	JN233705	115,300	*Klebsiella pneumoniae* ST258	[10]
IncFII(K)	97.97	CP000648	175,879	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578	Direct Submission
IncR	100	DQ449578	98,264	*Klebsiella pneumoniae* NK245	[11]

mostly clonal and geographically specific respectively. Although more than 90 different KPC variants, KPC-2 and -3 remain the most commonly identified variants. Interestingly, although KPCs are present in >100 different *K. pneumoniae* STs, the mass dissemination of KPC-producing *K. pneumoniae* has been primarily restricted to clone complex 258 (CC258), namely, ST258, ST11, ST340, and ST512 [2, 21, 22]. Though, the spread of KPC had been initiated by a single dominant clone sequence type 258 (ST258), it has now been reported among various STs across the World [23, 24]. While the ST258 is becoming the most prevalent carbapenem-resistant *K. pneumoniae* (CRKP) clone in North America, Latin America, and Europe [2, 25], however, in Asia, especially China, ST11 is the predominant clone (up to 60% of CRKP) [26, 27]. *K. pneumoniae* isolates belonged to the international high-risk clone ST147 form a cluster of closely related isolates and may share common resistance genes with the same or different alleles [28]. In one study, by resistome analysis of the eight ST147 isolates, the genes conferring resistance to penicillins ($bla_{\text{TEM-1}}$, $bla_{\text{SHV-11}}$, $bla_{\text{OXA-1}}$), cephalosporins ($bla_{\text{CTX-M-15}}$), carbapenems ($bla_{\text{OXA-48}}$), aminoglycosides [*aac(6′)Ib-cr*, *strA*, *strB*, *aacA4*], fluoroquinolones (*qnrS1, oqxAB*), fosfomycin (*fos*A), chloramphenicol (*flo*R), sulphonamides (*sul*1, *sul*2), tetracyclines (*tet*A) and trimethoprim (*dfr*A1) were found in ST147 and the tested antimicrobial susceptibility profiles of all isolates were in complete accordance with the genes from the resistome [29]. From these resistance genes, *fosA, oqxB, aac(a)-IIa,* $bla_{\text{TEM-1A}}$, bla_{SHV}, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$ are also common in *Kpn* Rize-53-TR.

There is no detailed data about the STs of *K. pneumoniae* identified from Turkey in the literature. However, when pathogen watch portal is analyzed, it is seen that whole genome sequence of 51 *K. pneumoniae* isolates from different MLST types was submitted between 2013 and 2014. Six of them belong to clone ST147, and they were obtained southern (Antalya and Kayseri) and south west (Izmir) of Turkey. The $bla_{\text{KPC-2}}$ gene could be detected in none of these six isolates and the others. Because, the KPC-2 carbapenemase was firstly identified in Turkey in 2014 in ST258 clonal group [30]. In one recent study from Turkey, a total of 93 carbapenem and colistin resistant *K. pneumoniae* isolates were analyzed, and two different MLSTs, ST101 (89/93) and ST147 (4/93) were determined [31]. The rate of ST147 was lower than ST101. In another study, $bla_{\text{KPC-3}}$ was firstly showed on *K. pneumoniae* ST147 with $bla_{\text{CTX-M-27}}$ and this ST147 strain also harbored $bla_{\text{SHV-11}}$, $bla_{\text{SHV-67}}$, and $bla_{\text{TEM-1}}$ [32].

CRKP is mainly caused by the spread of a few successful clones. Major representatives of these high-risk clonal lineages include ST11, ST15, ST307, ST17, ST37, ST101, and ST147 strains [33]. The KPC-2-producing *K. pneumoniae* ST147 is a globally successful clone and produces NDM, OXA-48, and CTX-M, as well as KPC. In the databases, there are 96 whole-genome sequences from *K. pneumoniae* in ST147 clonal group. When these genomes are examined in terms of the association of ST147 and KPC-2, there are only four isolates available [Canada (1), Italy (1) and Greek (2)] belonging to ST147 clonal group. However, while these four isolates belong to KL64/O2v1 serotype, the isolate in this study belongs to KL10/O3 serotype. Thus, both the first $bla_{\text{KPC-2}}$ gene on ST147 clonal group in Turkey and in KL10/O3 serotype in the World was determined. The phylogenetic tree was drawn according to the KL types of 96 *K. pneumoniae* from different countries, and the KL10/O3 serotype described in this study was located in the same clade from Bangladesh, India, Russia, Italy, Switzerland and Thailand. None of these carry KPC-2, and the dominant carbapenemase types appear to be primarily OXA-232, NDM-1/5/9 and OXA-48.

Dissemination of antibiotic resistance is driven by clonal expansion or horizontal gene transfer, including mainly mobile genetic elements (MGEs) [34, 35]. The extensive use of carbapenems has resulted in the evolution of plasmid-mediated carbapenemases, and *Klebsiella* turned out to be the major carbapenem-resistant Enterobacterales (CRE) worldwide [36]. The $bla_{\text{KPC-2}}$ may be found on plasmids from different incompatibility replicon groups, such as IncF, IncI2, IncX, IncA/C, IncR, and ColE1. CC258 was determined the type responsible for the pandemic spread of KPC-Kp on the World [37]. Different KPC-containing plasmids have also been identified in CC258 [38], but the most predominant plasmid type is incompatibility group F plasmids that harbor several FII replicons (IncFII-like plasmids) [39]. IncFIIK replicon is the preferred one [23]. This is well exemplified by the global spread of the KPC carbapenemase involving the incompatibility group FIIk (IncFIIk) plasmids in *K. pneumoniae* [21]. Chmelnitsky et al. proposed that the successful global dissemination and survival of ST258 were, in part, dependent on the combination of $bla_{\text{KPC-2}}$ on IncF plasmids with factors inherently present on the chromosome of this high-risk clone [40]. In our data, the same is true for ST147. In conclusion, IncFII(K) plasmid harboring $bla_{\text{KPC-2}}$ contributes the presence in *K. pneumoniae* clinical isolate in Turkey and transmitted it in clinical environments.

The genome sequence of *Kpn* Rize-53-TR shows that this clinical isolate is equipped with important antibiotic resistance genes. This gives insight into the history of its long journey. The fact that it contains five different plasmids and many antibiotic resistance genes that provide resistance to commonly used antibiotic classes, shows that the isolate has a long history in the pathogenicity of *K. pneumoniae*. To obtain these plasmids or genes, it shared the same environment with many of its comrades, spent time together and had the opportunity to exchange genetic information. It is not possible to know the deaths, the processes and costs of the diseases during this period.

CONCLUSION

This study found that the isolate *Kpn* Rize-53-TR was resistant to all group β-lactams and carbapenems. Seven different β-lactamase from class-A ($bla_{\text{KPC-2}}$, $bla_{\text{TEM-1A}}$, $bla_{\text{SHV-67}}$, $bla_{\text{CTX-M-15}}$) and D ($bla_{\text{OXA-1-2-9}}$) were determined. The mutation in *gyrA* and *parC* genes were

responsible for the resistance against quinolone. The bla_{KPC-2} was firtly found in both ST147 clonal group in Turkey and in serotype O3/O3a in the world. Five plasmids IncFII(K), Col(BS512), IncR, IncFIA(HI1) and IncFIB(pQil) were determined in *Kpn* Rize-53-TR and bla_{KPC-2} was on IncFII(K) plasmid. Observation of KPC-2 in a new serotype, that is becoming carrier of multiple antibiotic resistance genes makes the *K. pneumoniae* strain described in this study more pathogenic and complicates its treatment.

Conflict of interests: None.

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