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RESEARCH ARTICLE

Emergence of carbapenemase-producing and colistin resistant *Klebsiella pneumoniae* ST101 high-risk clone in Turkey

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ABSTRACT

Carbapenemase-producing and colistin resistant *Klebsiella pneumoniae* has become a worldwide healthcare problem. This study describes molecular characterization of carbapenemase-producing and colistin resistant clinical *K. pneumoniae* isolates.

A total of 93 non-replicate carbapenem and colistin resistant *K. pneumoniae* were recovered from clinical specimens in a university hospital during 2017–2019. Detection of $bla_{\text{OXA-48}}$, bla_{KPC}, $bla_{\text{NDM-1}}$, bla_{IMP}, $bla_{\text{VIM-1}}$ and *mcr-1*, *-2*, *-3*, *-4*, *-5*, *-6*, *-7*, and *-8* genes was performed by PCR. The bacterial isolates were assigned to clonal lineages by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

All isolates harbored $bla_{\text{OXA-48}}$ and only two isolates harbored $bla_{\text{OXA-48}}$, and $bla_{\text{NDM-1}}$ genes together. In colistin resistant *K. pneumoniae*, *mcr-1* was detected in two (2.1%) isolates. Ninety three isolates of *K. pneumoniae* were categorized into three clusters and five pulsotypes. MLST revealed two different sequence types, ST101 (89/93) and ST147 (4/93).

In our study ST101 was found to be a significantly dominant clone carrying $bla_{\text{OXA-48}}$ and among our strains a low frequency of *mcr-1* gene was determined. The emergence of colistin resistance was observed in *K. pneumoniae* ST101 isolates. ST101 may become a global threat in the dissemination of carbapenem and colistin resistance.

KEYWORDS

Klebsiella pneumoniae, carbapenemase, colistin resistance, *mcr-1*, $bla_{\text{OXA-48}}$, PFGE, MLST, ST101, ST147

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen which can cause different types of healthcare-associated infections. Enhanced use of carbapenems in clinical practice, promoted emergence of carbapenem-resistant *K. pneumoniae* (CRKP) worldwide in recent years [1]. CRKP has mainly been link to *K. pneumoniae* carbapenemase (KPC), oxacillinase-48 (OXA-48), and metallo-β-lactamases (MBLs), such as NDM, IMP, and VIM type carbapenemases. While these plasmid-encoded carbapenemases have been increasingly reported worldwide, their prevalence varies geographically [2]. The first identified OXA-48 producer was a *K. pneumoniae* strain isolated in Turkey in 2003 [3]. Since then, OXA-48 producers have been extensively reported from Turkey as a source of nosocomial outbreaks [4–8]. Worldwide distribution of OXA-48 now includes countries in Europe, in the southern and eastern part of the Mediterranean Sea, and Africa [3–8].

Treatment of infected patients with CRKP is always problematic due to their multidrug resistance phenotype, and several therapeutic options have been considered. Colistin is one of these therapeutic options. However, colistin resistance had been observed in CRKP isolates,

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and rapid dissemination of colistin resistant isolates has been recently reported [9]. Colistin resistance mechanisms are presumed to be linked to chromosomal mutation untransferable via horizontal gene transfer [10]. Recently, several plasmid-mediated colistin resistance genes, named *mcr*, encoding pEtN transferases, have also been reported in *K. pneumoniae* [11].

Dissemination of CRKP is mainly caused by the spread of a few successful clones. Major representatives of these high-risk clonal lineages include sequence type (ST) 11, ST15, ST307, ST17, ST37, ST101, and ST147 strains [1]. ST258 strains are major players in the worldwide spread of KPC-type carbapenemases, and are responsible for 68% of the CRKP outbreaks [12]. ST101 strains harbor different clinically-relevant resistance determinants, such as carbapenemases of the KPC, OXA-48, VIM, and NDM types [1].

In this study, carbapenemase producing and colistin resistant clinical *K.pneumoniae* isolates were characterized to evaluate genetic differences and relationships, and prevalence of carbapenem resistance determinants, as well as to determine plasmid-mediated colistin resistance mechanism.

MATERIAL AND METHODS

Bacterial isolates and susceptibility testing

We retrospectively analyzed ninety-three carbapenem and colistin resistant *K. pneumoniae* isolates consecutively isolated from patients who were hospitalized at the Hacettepe University Hospitals between October 2017 and December 2019. The Hacettepe University Hospitals are tertiary care hospitals of 1,040 beds that provides specialized attention to a population size of ~5.504 million habitants in the capital of Turkey. In the period between October 2017 and December 2019, altogether 8624 *K. pneumoniae* isolates were obtained from routine microbiological cultures of clinical samples. In total, 93 carbapenem and colistin resistant *K. pneumoniae* isolates were obtained. These isolates were detected from blood (n = 34), urine (n = 26), abscess (n = 13), tracheal aspirate (n = 11), peritoneal fluid (n = 4), cerebrospinal fluid (n = 2), synovial fluid (n = 1), pleural fluid (n = 1), and pericardial fluid (n = 1). Isolates were identified with conventional tests (Gram staining, catalase and oxidase tests), and matrix assisted lazer desorption ionization time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Germany). All isolates were identified with a score ≥2.0; accurate identification to the species level by MALDI-TOF MS. Antibiotic susceptibility profiles of isolates were determined by BD Phoenix™ automated susceptibility testing system (Becton Dickinson and Company BD, USA). Isolates non-susceptible to at least one carbapenem (ertapenem, meropenem, and imipenem) were also tested for carbapenem resistance by gradient test (bioMérieux, France) according to manufacturer's instructions. CRKP was defined when the isolate was resistant to ertapenem, meropenem or imipenem by gradient test. Colistin MICs were determined for 93 CRKP isolates using the Sensititre™ plate (Thermo Fisher, UK). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoints [13]. *Escherichia coli* ATCC 25922 and *E. coli* NCTC 13846 (*mcr-1* positive) was used for quality control. The isolates were stored in tryptic soy broth containing 10% (v/v) glycerol at −80 °C until use.

Molecular analysis of carbapenem and colistin resistance

Genomic DNA was isolated using the QIAsymphony DSP Virus/Pathogen kit in the QIAsymphony system according to the manufacturer's instructions (Qiagen, USA). OXA-48, KPC, NDM-1, IMP, and VIM-1 carbapenemases were identified by PCR amplification and sequencing as described previously [14]. The colistin resistant isolates were screened by simplex PCRs for the presence of *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5, mcr-6*, mcr-7, and *mcr-8* genes [15–21] (Supplementary Table S1).

PFGE

Pulsed-field gel electrophoresis (PFGE) was performed as per a method described in a previous study [22]. A thin slice of plug was digested overnight at 37 °C with 50 U of the XbaI restriction enzyme according to the manufacturer's instructions. The restriction fragments were separated through PFGE in 1% agarose gel (Bio-Rad, USA) with 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, and 1.0 mM EDTA [pH 8.0]) for 22 h at 200 V and 14 °C, with ramp times of 2 s-40 s using the CHEF Mapper apparatus (Bio-Rad, USA). The gels were stained in ethidium bromide (1 mg/mL), were viewed under an ultra-violet trans-illumination. Digital images were stored electronically. PFGE banding patterns were analysed with the BioNumerics Software (Applied Maths, Belgium) using the dice coefficient and unweighted pair group method with arithmetic averages algorithm. PFGE patterns were compared and analysed according to the criteria mentioned by Tenover et al. [23].

MLST

Multilocus sequence typing (MLST) was performed on *K. pneumoniae* isolates according to the protocol described on the *K. pneumoniae* MLST website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Seven conserved housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) were used [24]. MLST results were typed according to the updated international *K. pneumoniae* MLST database at the Pasteur Institute in Paris, France.

RESULTS

Bacterial isolates and susceptibility testing

In the period between October 2017 and December 2019, a total of 8,624 clinical isolates of *K. pneumoniae* were isolated from patients admitted to Hacettepe University Hospitals. Among those, 2,259 isolates (26.2%) were non-susceptible to

Table 1. MIC (μg/mL) profiles of carbapenem and colistin resistant *K. pneumoniae* isolates

	2017 (n = 18)	2018 (n = 38)	2019 (n = 37)
Colistin			
MIC range	4–64	4–128	4–64
MIC_{50}	4	4	4
MIC_{90}	16	32	64
Meropenem			
MIC range	16–128	16–128	16–256
MIC_{50}	16	16	16
MIC_{90}	32	64	128
Imipenem			
MIC range	16–64	16–128	8–64
MIC_{50}	16	16	8
MIC_{90}	32	64	32
Ertapenem			
MIC range	2–64	2–128	2–64
MIC_{50}	2	16	16
MIC_{90}	32	32	32

at least one carbapenem by gradient test and were tested for colistin resistance. A total of 93 isolates (4.1%) out of this subset showed a colistin resistant phenotype by Sensititre with minimum inhibitory concentrations (MIC) that ranged between 4 and 128 μg/m. Carbapenem and colistin MIC ranges, MIC_{50} and MIC_{90} profiles of the isolates are summarized in Table 1. Overall carbapenem and colistin resistance rates are shown in Supplementary Table S2.

Molecular analysis of carbapenem and colistin resistance

The PCR results indicated that $bla_{\text{OXA-48}}$ gene was detected in all *K. pneumoniae* isolates (100%), and 2.1% (n = 2) of the isolates co-produced $bla_{\text{OXA-48}}$, and $bla_{\text{NDM-1}}$. Other tested carbapenemase genes, such as bla_{KPC}, bla_{IMP}, and $bla_{\text{VIM-1}}$ could not detected in any of the isolates. Detection of *mcr 1-8* genes using PCR technique revealed that two (2.1%) isolates were positive for *mcr-1*. No *mcr-2, -3, 4, -5, -6, -7,* and *-8* were detected among all tested isolates.

PFGE

The characteristics of the molecular epidemiology of the 93 carbapenem and colistin resistant *K. pneumoniae* isolates are displayed in Fig. 1. All the 93 carbapenem and colistin resistant *K. pneumoniae* isolates were grouped into three cluster and five pulsotypes. Cluster three is the largest cluster that possesses 71 isolates. Fourteen isolates belonged to cluster one and eight isolates belong to cluster two. PFGE discriminatory power was of 96%, as calculated by Simpson's Index of Diversity [25].

MLST

Ninety-three carbapenem and colistin resistant *K. pneumoniae* isolates were analysed by MLST, and two ST types were detected. ST101(95.6%, 89/93) was the dominant ST type followed by ST147 (4.4%, 4/93). Among four isolates of ST147, two isolates carried both $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ genes, and the other two ST147 clones coproduce $bla_{\text{OXA-48}}$ and *mcr-1* genes (Fig. 1).

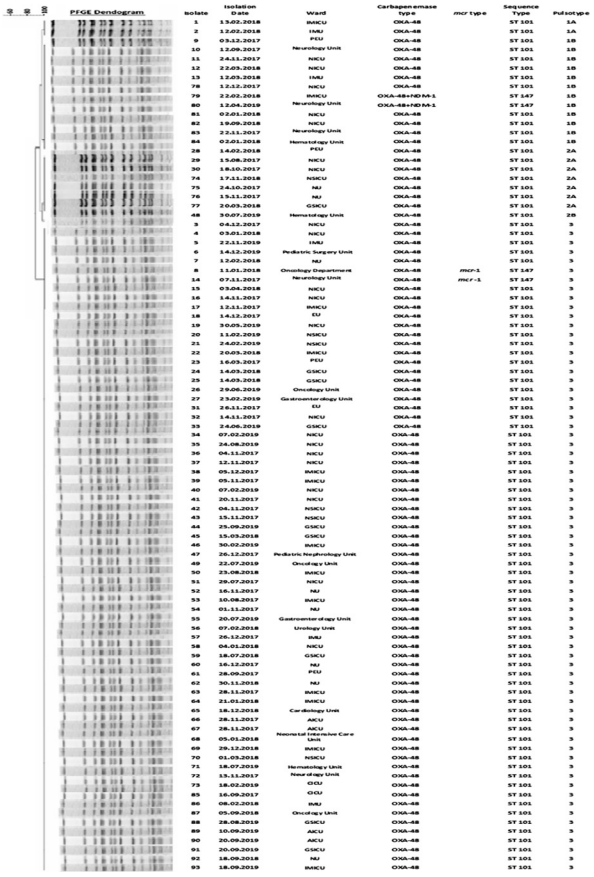

Fig. 1. Dendrogram based on pulsed-field gel electrophoresis pattern analysis (PFGE) of 93 colistin and carbapenem resistant *K. pneumoniae* isolates from different wardss and their ST determined via multilocus sequence typing (MLST)

DISCUSSION

Multidrug resistant pathogens have become a global problem recently [12]. *K. pneumoniae* is an important nosocomial multidrug resistant pathogen that can cause high morbidity and mortality [26]. After widespread dissemination of carbapenemase producing *K. pneumoniae* isolates; colistin resistance has emerged in *K. pneumoniae* isolates and caused problems in treatment modalities [9, 26, 27]. CRKP isolates that produce carbapenemases such as the OXA-48, KPC, VIM-1, NDM-1, and IMP, have been reported worldwide [1, 3, 5–7, 9, 10, 26]. KPCs are the most clinically common enzymes, and have been detected in North America (especially the United States), South America (Colombia, Argentina), Europe (Greece, Italy, Poland), Asia (China), and the Middle East (Israel) [28–30]. Turkey is a country with a specific epidemiology, where OXA-48 carbapenemase has been extensively identified. However,

first KPC-2-positive *K. pneumoniae* have been identified in Turkey, in 2014 [31]. Since then, sporadic KPC-producing *K. pneumoniae* was reported [32, 33]. In the present study, we didn't detect bla_{KPC} gene among tested carbapenem and colistin resistant *K. pneumoniae* isolates.

MBLs identified in *K. pneumoniae* mainly reported from Japan (IMP), Taiwan (IMP), Indian subcontinent (NDM), Balkan states (NDM), and Greece (VIM) [33]. Recent findings suggest that the Balkan states and the Middle East may act as secondary reservoirs of NDM-1 producers. In the European Survey of Carbapenemase-Producing Enterobacteriaceae (EuSCAPE) study, Turkey was classified as a stage 3 country on a scale of 1–5 (1: no reported case; 5: endemic situation) for the existence of NDM carbapenemases. Among CRKP, NDM-1 carbapenemases detected between 6.3% and 52.9% in our country [34, 35]. Moreover, the coproduction of both OXA-48 and NDM-1carbapenemases has been frequently reported. We detected two (2.1%) NDM-1-producing isolates, which have already been found to harbour OXA-48 carbapenemases. Interestingly, NDM-1 carbapenemase detected in a very low rate compared to our country results. Our hospital setting is not an endemic region for bla_{NDM-1} positive *K. pneumoniae.* The carbapenemase genes bla_{IMP} and bla_{VIM} were reported in a low but notable incidence in Turkey like other countries [27, 35–38]. In our study, bla_{IMP} and bla_{VIM} genes were not detected in any of the isolates.

OXA-48-producing *K. pneumoniae* was first reported from Turkey and it is endemic for our country [30, 32, 36, 37]. The emergence of the OXA-48 enzyme is mediated by the rapid spread of a broad host-range conjugative plasmid harboring the *bla*OXA-48 gene. Plasmid harboring *bla*OXA-48 with the Tn1999.2 transposon detected from a *K. pneumoniae* isolate in Turkey [39]. OXA-48-producing *K. pneumoniae* is also endemic in certain North African and European countries (Morocco, Tunisia, Spain, Belgium) [40]. OXA-48-producing *K. pneumoniae* remain relatively uncommon in the United States and Canada [41]. As it was expected, we detected bla_{OXA-48} gene in all tested CRKP isolates. However, two of these isolates were positive for both bla_{NDM-1} and bla_{OXA-48} genes.

Multilocus sequence typing is an excellent method in evolutionary studies for exploring the common ancestral lineages of bacterial isolates [20]. Various ST types (ST11, ST14, ST101, ST147, and ST258) and resistance mechanisms can be related to carbapenem and colistin resistance in *K. pneumoniae* [41]. A single *K. pneumoniae* clone ST258 was identified extensively worldwide, indicating that it may have contributed to the spread of the bla_{KPC} genes [26]. On the other hand, KPC-producing *K. pneumoniae* remains rare for our country [31, 32]. Among all tested isolates, we didn't detect KPC-producing *K. pneumoniae* and also its emerging high-risk clone ST258. We found that the epidemic *Klebsiella pneumonia* isolate in our hospital was in ST101 type. ST101 was previously accepted as a high-risk epidemic clone, and it was reported that the ST101 clone was associated with various β-lactamases, including NDM-1, OXA-48, and CTX-M-15 [38]. Nevertheless, CRKP assigned to ST101 are carbapenem resistant frequently because of the production OXA-48. David et al., analysed the genome sequences of *K. pneumoniae* strains, isolated from patients in 244 hospitals in 32 countries during the European Survey of Carbapenemase-Producing Enterobacteriaceae. CRKP are concentrated in four clonal lineages, ST11, ST15, ST101, ST258/512, and authors identified OXA-48-producing ST101 clones in Romania, Spain, and Turkey [39]. Also, the emergence of colistin resistance has been observed in CRKP isolates, and colistin resistance was shown to be associated with the ST101 clone. Detection of carbapenem and colistin resistant *K. pneumoniae* ST101 was reported from Italy and Serbia [42, 43]. A large multicentre cohort study, describe the molecular characteristics of clinical colistin and carbapenem resistant *K. pneumoniae* isolates. Researchers observed a significant association between ST101, OXA-48, and colistin resistance [44]. Our study reports the clonal expansion of emerging ST101 clone associated with OXA-48 producing and colistin resistance in our hospital settings.

K. pneumoniae ST147 is an emerging high-risk clone that was first identified in Greece and has been associated with VIM and KPC carbapenemases in that country [45]. This global ST has also been associated with NDM and OXA-181 carbapenemases in various countries, including Switzerland, Iraq, Canada, United Kingdom, India, and Italy [26, 46]. In the current study, two isolates of ST147 were detected which co-harbored bla_{NDM-1} and bla_{OXA-48} genes.

Carbapenem-resistance among *K. pneumoniae* isolates makes colistin the last therapeutic option for the treatment. With the rise in consumption of colistin, cases of colistin resistant CRKP isolates are reported globally [1, 9, 11]. Chromosomal mutations in *phoP/phoQ, pmrA/pmrB, mgrB* and plasmid-borne mobile colistin resistance genes (*mcr-1* to *mcr-9*) positivity have an important role in increasing colistin resistance in *K. pneumoniae* [47, 10, 11]. The highest colistin resistance rate was reported in Asia (especially Korea and Singapore), followed by Europe (especially Greece) and America, where colistin resistance rates are continually increasing [48]. Nowadays, all known *mcr* genes have been detected in various *K. pneumoniae* isolates, whereas a small number of studies have shown the presence of *mcr* genes in clinical *K. pneumoniae* isolates [49]. Several studies suggested that chromosomal mutations rather than *mcr* genes positivity might have an important role in colistin resistance [47, 44, 50]. Different STs such as ST274, ST461, ST15, ST16, ST416, ST1890, ST37, ST1942, ST101, ST147, ST258, ST152, and ST15 were detected in carbapenem and colistin resistant *K. pneumoniae* [42, 50, 51]. We detected two ST types, ST101 (95.6%) and ST147 (4.4%) in carbapenem and colistin resistant *K. pneumoniae.* In Turkey, a few study investigated *mcr-1* to *-3* genes among carbapenem and colistin resistant *K. pneumoniae* isolates and only Arabacı et al., determined *mcr-1* positive KRCP (5.2%) [44, 51–53]. In this study, we investigated *mcr-1* to *-8* genes in colistin and carbapenem resistant *K. pneumoniae* and reported the molecular characteristics of *mcr-1* positive CRKP. To our knowledge, this is the first report of *mcr-1* positive *K. pneumoniae* isolates that produce both NDM-1 and OXA-48 while also belonging to the one of the emerging clones ST147 from Turkey.

CONCLUSION

We identified two different STs, namely, ST101 and ST147 among the carbapenem and colistin resistant *K. pneumoniae* isolates identified during 2017 and 2019. ST101 is a epidemic ST and has been associated with OXA-48. Our results show that this ST also has the ability to develop colistin resistance. Early detection and surveillance can prevent the spread of carbapenem and colistin resistant *K. pneumoniae* isolates.

Ethical committee approval: Not required.

Conflict of interest: The authors declare no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1556/030.2020.01275.

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