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Original Article

Molecular epidemiological investigation of carbapenem resistant *Klebsiella pneumoniae* isolated from intensive care unit patients of six geographical regions of Turkey

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Abstract

Introduction: *Klebsiella pneumonia* causes serious infections in hospitalized patients. In recent years, carbapenem-resistant infections increased in the world. The molecular epidemiological investigation of carbapenem-resistant *K. pneumoniae* isolates was aimed in this study.

Methodology: Fifty carbapenem-resistant *K. pneumoniae* isolates from six geographical regions of Turkey between September 2019-2020 were included in the study. The disk diffusion method was used for the antibiotic susceptibility testing. The microdilution confirmed colistin susceptibility. Genetic diversity was investigated by MLST (Multi-Locus Sequence Typing).

Results: The resistance rates were as follows: 49 (98%) for meropenem, 47 (94%) imipenem, 50 (100%) ertapenem, 30 (60%) colistin and amoxicillin-clavulanate, 49 (98%) ceftriaxone, 48 (96%) cefepime, 50 (100%) piperacillin-tazobactam, 47 (94%) ciprofloxacin, 40 (80%) amikacin, 37 (74%) gentamicin. An isolate resistant to colistin by disk diffusion was found as susceptible to microdilution. ST 2096 was the most common (n:16) sequence type by MLST.

ST 101 (n:7), ST14 (n:6), ST 147 and ST 15 (n:4), ST391 (n:3), ST 377 and ST16 (n:2), ST22, ST 307, ST 985, ST 336, ST 345, and ST 3681 (n:1) were classified in other isolates. In İstanbul and Ankara ST2096 was common. Among Turkey isolates, the most common clonal complexes (CC) were CC14 (n:26) and CC11 (n = 7).

Conclusions: In Turkey, a polyclonal population of CC14 throughout the country and inter-hospital spread were indicated. The use of molecular typing tools will highlight understanding the transmission dynamics.

Key words: *K. pneumonia;* MLST; carbapenem; colistin.

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Introduction

Klebsiella pneumoniae causes serious infections such as pneumonia, and bloodstream and urinary tract infections in immunocompromised individuals, long-term hospitalized patients, and neonates [1]. With the detection of extended-spectrum β-lactamases (ESBL), the use of carbapenems for the *K. pneumoniae* infections increased [2]. In 1996, the emergence of carbapenem-resistant isolates caused the limitation of antibiotic therapy options [1].

Carbapenem resistance is mediated by two main mechanisms including reduction of membrane permeability in the bacterial cell wall and the production of carbapenemases. Carbapenemases are

classified in class A (*K. pneumoniae* carbapenemase, KPC), class B (metallo-β-lactamases) and class D oxacillinases according to Ambler classification [3,4].

Currently, carbapenem-resistant *K. pneumoniae* is a global threat causing mortality in hospitalized patients and intensive care unit patients. A systematic review and meta-analysis reported that while mortality was 42.14% in carbapenem-resistant *K. pneumoniae* infections, 21.16% in carbapenem-susceptible *K. pneumoniae* infections [1].

Colistin is one of the drugs of last choice in the treatment of carbapenem-resistant infections. However, in recent years, plasmid-mediated colistin resistance caused by *mcr-1* gene and its variants emerged among *K. pneumonia* isolates and caused outbreaks in hospital settings [1,5]. For the prevention and controlling of carbapenem/colistin resistant *K. pneumonia* infections, accurate identification of infections, surveillance studies, the determination of clonal relationship between isolates by molecular epidemiological tools were required [6]. In this study, carbapenem-resistant *K. pneumoniae* blood culture isolates of intensive care unit patients in six regions of Turkey were analysed by Multi-Locus Sequence Typing (MLST) method and STs and clonal relationship of isolates circulating in Turkish hospitals were determined.

Methodology

A total of 50 carbapenem-resistant *K. pneumoniae* isolates recovered from blood cultures of intensive care unit patients from six geographical regions of Turkey between September 2019 and September 2020 were included in this study. Samples isolated from patients hospitalized in the same ward and samples belonging to the same patients were excluded from the study. Fifteen *K. pneumoniae* isolates from Istanbul, 5 from Ankara, 7 from Sivas, 11 from Diyarbakır, 7 from İzmir, and 5 from Isparta were collected. Antibiograms of the isolates were performed by disc diffusion method, VITEK 2 Compact (Bio-Merieux, France) systems, and/or E-test methods in each hospital. *Klebsiella pneumoniae* isolates resistant to at least one of the carbapenems during the study period were sent to Ankara Yıldırım Beyazıt University Medical Microbiology Laboratory. The antibiotic susceptibility results and demographic data were reported from each hospital. The following procedures were continued in Ankara Yıldırım Beyazıt University Medical Microbiology Laboratory.

The susceptibility of the isolates to colistin was confirmed by the microdilution method. Antibiogram results were evaluated according to the recommendations of EUCAST (European Committee on Antimicrobial Susceptibility Testing) [7].

For MLST analysis, firstly, the isolates were inoculated on Mueller Hinton agar medium and incubated at 37 °C overnight. Genomic DNA isolation was performed with Gene Matrix Tissue & Bacterial DNA Purification Kit (EURx, Gdańsk, Poland) in accordance with the manufacturer's instructions. DNAs were stored at -20 °C until further use. Then, seven house-keeping genes (rpoB, gapA, mdh, pgi, phoE, infB, tonB) for each *K. pneumoniae* isolate were amplified by PCR (polymerase chain reaction) using 5X Firepol Master mix kit (Solis Biodyne, Tartu, Estonia). The primer sequences and PCR product lengths used are shown in Table 1. PCR amplification was performed by using an initial denaturation at 94 °C for 2 minutes, followed by 35 cycles at 94 °C for 20 seconds, at 50 °C or 60 °C for 30 seconds, at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes [8]. The binding temperature was set to 60 °C for mdh, pgi, and gapA PCRs and 50 °C for others. The obtained PCR samples were then sequenced using the Sanger method, and the sequence of the genes was taken as

Table 1. Primer sequences and expected PCR product lengths [10].

rpoB	F: GGCGAAATGGCWGAGAACCA R: GAGTCTTCGAAGTTGTAACC	1075bp*
gapA	F: TGAAATATGACTCCACTCACGG R: CTTCAGAAGCGGCTTTGATGGCTT	662 bp
mdh	F: CCCAACTCGCTTCAGGTTCAG R: CCGTTTTTCCCCAGCAGCAG	756bp
pgi	F: GAGAAAAACCTGCCTGTACTGCTGGC R: CGCGCCACGCTTTATAGCGGTTAAT	566b p
phoE	F: ACCTACCGCAACACCGACTTCTTCGG R: TGATCAGAACTGGTAGGTGAT	602 bp
infB	F: CTCGCTGCTGGACTATATTCG R: CGCTTTCAGCTCAAGAACTTC	462 bp
tonB	F: CTTTATACCTCGGTACATCAGGTT R: ATTCGCCGGCTGRGCRGAGAG	539 bp

*base pair.

fasta. Then, these sequences were analysed in the Institute Pasteur MLST database (https://bigsdb.web.pasteur.fr/index.html) to generate allele numbers and sequence type (ST) numbers for each gene of the strains.

Results

Findings of the patients

The age of the intensive care unit patients was between 1-86 years. Twenty-eight (56%) of the patients were male and 22 (44%) were female.

Antibiogram results

According to the antibiogram results reported in Turkey hospitals, the resistant rates of isolates were 98% (n:49) for meropenem, 94% (n:47) for imipenem,

Figure 1. Antibiotic-resistant rates of isolates according to antibiogram results.

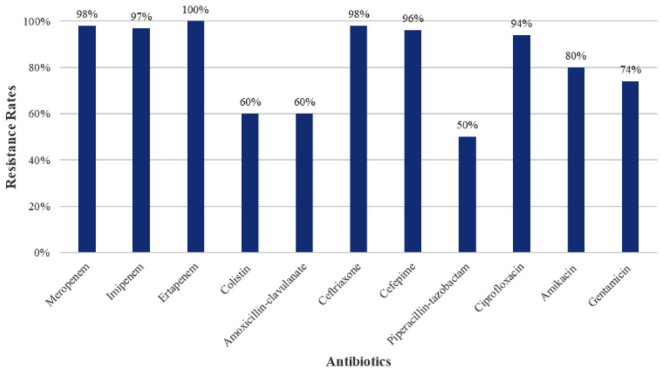


Table 2. ST types of *K.pneumoniae* isolates and their distribution between cities.

NO	Pasteur MLST ID	City	gapA	infB	mdh	pgi	phoE	rpoB	tonB	ST	CC*
1	16656	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
2	16657	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
3	16658	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
4	16659	Istanbul	2	6	1	5	4	1	6	**101**	**11**
5	16660	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
6	16661	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
7	16662	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
8	16663	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
9	16664	Istanbul	3	4	6	1	7	4	38	**147**	
10	16665	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
11	16666	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
12	16667	Istanbul	3	4	6	1	7	4	38	**147**	
13	16668	Istanbul	3	4	6	1	7	4	38	**147**	
14	16669	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
15	16670	Istanbul	1	6	1	1	1	46	1	**2096**	**14**
16	16671	Ankara	1	6	1	1	1	46	1	**2096**	**14**
17	16672	Ankara	1	6	1	1	1	46	1	**2096**	**14**
18	16673	Ankara	1	6	1	1	1	46	1	**2096**	**14**
19	16674	Ankara	1	6	1	1	1	46	1	**2096**	**14**
20	16675	Ankara	1	6	1	1	1	46	1	**2096**	**14**
21	16676	Sivas	1	1	1	1	1	1	1	**15**	**14**
22	16677	Sivas	3	4	6	1	7	4	38	**147**	
23	16678	Sivas	2	1	2	1	4	4	4	**16**	
24	16679	Sivas	1	1	1	1	1	1	1	**15**	**14**
25	16680	Sivas	1	1	1	1	1	1	1	**15**	**14**
26	16681	Sivas	10	3	2	2	6	4	4	**985**	
27	16682	Sivas	1	1	1	1	1	1	1	**15**	**14**
28	16683	Diyarbakır	10	5	1	4	6	4	4	**391**	
29	16684	Diyarbakır	1	6	1	1	1	202	1	**3681**	**14**
30	16685	Diyarbakır	2	6	1	5	4	1	6	**101**	**11**
31	16686	Diyarbakır	2	6	1	5	4	1	6	**101**	**11**
32	16687	Diyarbakır	2	1	2	1	4	4	4	**16**	
33	16688	Diyarbakır	2	1	5	2	4	4	12	**345**	
34	16689	Diyarbakır	10	5	1	4	6	4	4	**391**	
35	16690	Diyarbakır	2	1	1	1	72	4	4	**336**	
36	16691	Diyarbakır	2	6	1	5	4	1	6	**101**	**11**
37	16692	Diyarbakır	10	5	1	4	6	4	4	**391**	
38	16693	Diyarbakır	2	3	1	1	1	4	4	**22**	
39	16694	İzmir	1	6	1	1	1	1	1	**14**	**14**
40	16695	İzmir	1	6	1	1	1	1	1	**14**	**14**
41	16696	İzmir	1	6	1	1	1	1	1	**14**	**14**
42	16697	İzmir	4	1	2	52	1	1	7	**307**	
43	16698	İzmir	1	6	1	1	1	1	1	**14**	**14**
44	16699	İzmir	1	6	1	1	1	1	1	**14**	**14**
45	16700	İzmir	1	6	1	1	1	1	1	**14**	**14**
46	16701	Isparta	10	20	2	1	9	11	12	**377**	
47	16702	Isparta	2	6	1	5	4	1	6	**101**	**11**
48	16703	Isparta	2	6	1	5	4	1	6	**101**	**11**
49	16704	Isparta	2	6	1	5	4	1	6	**101**	**11**
50	16705	Isparta	10	20	2	1	9	11	12	**377**	

*CC clonal complex.

100 % (n:50) for ertapenem, 60% (n:30) for colistin and 60% (n:30) amoxicillin-clavulanate (Figure 1). The resistance rates to ceftriaxone, cefepime, piperacillin-tazobactam, ciprofloxacin, amikacin, and gentamicin were 98%, 96%, 50%, 94%, 80%, and 74%, respectively (Figure 1). An isolate that was detected to be resistant to colistin by the automated system was found to be susceptible to colistin by microdilution.

MLST Results

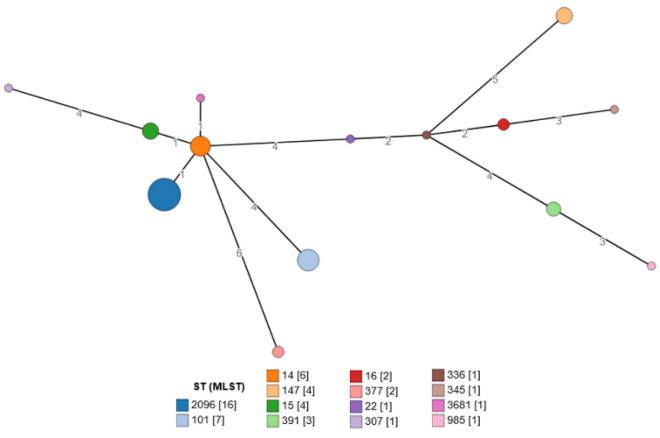
A total of 14 different STs were found by MLST of *K. pneumoniae* isolates using gapA, infB, mdh, pgi, phoE, rpoB, tonB primers. The ST 2096 was the most common type detected in 16 isolates followed by ST 101 (n:7), ST14 (n:6), ST 147 (n:4), ST 15 (n:4), ST391 (n:3), ST 377 (n:2), and ST16 (n:2), ST22 (n:1), ST 307 (n:1), ST 985 (n:1), ST 336 (n:1), ST 345 (n:1), ST 3681 (n:1). In Istanbul and Ankara which are the most crowded cities of Turkey ST2096 was common. In Sivas ST15, in Diyarbakır ST391 and ST101 in İzmir ST14, in Isparta ST101 were the most common STs.

Among Turkey isolates, the most common clonal complex (CC) was CC14 (n:26) and matched the central genotype at ≥ 4 loci. The second most common isolates were detected as CC 11 (n:7) (Table 2).

The Minimum spanning tree showed the heterogeneity of the STs in 50 *K. pneumoniae* isolates (Figure 2).

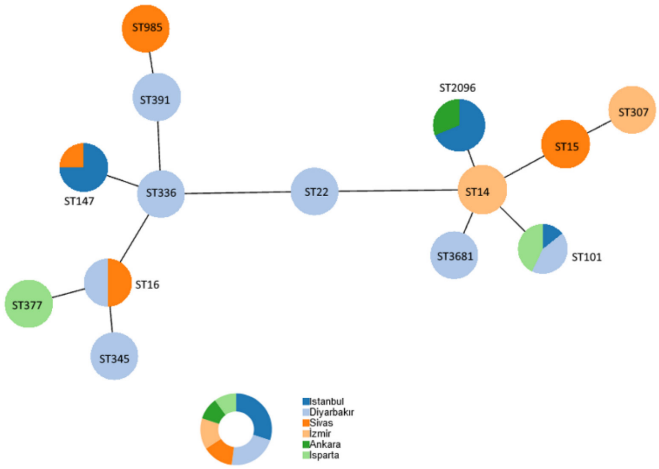
STs detected in ≥ 2 different cities were ST101, ST2096, ST147, and ST16. Each of the remaining 10 STs was found in any one of the five provinces (Figure 3).

Figure 2. Minimum spanning tree analysis of 50 carbapenem and/or colistin-resistant K. pneumoniae isolates based on allelic profiles of MLSTs.



Each colour represents an ST; STs are also numbered and the number of the strains in each ST was shown in brackets. The size of circles indicates the number of isolates within the related STs.

Figure 3. Distribution of the 14 STs of the 50 K pneumoniae isolates by cities.



There is no common ST detected in all centres. STs found in ≥ 2 different cities were ST101, ST2096, ST147, and ST16.

Discussion

Carbapenem resistant *Klebsiella pneumoniae* infections are a global threat. The risk factors for colonization and infection of carbapenem-resistant *Klebsiella pneumoniae* were comorbid diseases, long hospital stays, invasive medical device use, mechanical ventilation, and inappropriate antibiotic treatments [10]. In a meta-analysis, mortality rate in hospitalized patients (n:2462) with bloodstream infection and urinary tract infection caused by carbapenem-resistant *K. pneumoniae*, were 54.30% and 13.52%, respectively. The mortality rates reported in studies from North America, South America, Europe, and Asia were 33.24%, 46.71%, 50.06%, and 44.82%, respectively in carbapenem-resistant *K. pneumoniae* bloodstream infections [1,9]. Carbapenem-resistant isolates have high resistance rates to antibiotics other than carbapenems. In Turkey, carbapenem-resistant isolates were also problematic. In a study conducted in 57 carbapenem-resistant *K. pneumoniae* hospitalized patient isolates in Istanbul, Turkey, antibiotic resistance rates were reported as 52.63% for amikacin; 73.69% for trimethoprim sulfamethoxazole; 91.23% for cefepime; 82.46% for tigecycline; 59.65% for colistin. In the same study, carbapenemase positivity was 82.45% for blaOXA-48, 40.35% for blaOXA-55 and blaOXA-51, blaOXA-23, blaOXA-24, blaIMP were also detected in carbapenem-resistant *K.pneumoniae* isolates. The *mcr-1* gene responsible for the plasmid-mediated spread of colistin resistance was also found in three isolates [10]. In current study, the resistance rates for meropenem, imipenem, ertapenem, colistin, amoxicillin-

clavulanate, ceftriaxone, cefepime, piperacillin-tazobactam, ciprofloxacin, amikacin and gentamicin were as 98%, 94%, 100%, 60%, 100%, 98%, 96%, 100%, 94%, 80%, and 74%, respectively. These findings indicated that controlling the spread of horizontal antibiotic resistance genes was an emergency in Turkey and in the world [1,9,10].

In the current study and literature, incompatibilities were reported between the automated system and microdilution method. It was demonstrated the need for the colistin antibiogram confirmation by microdilution method [10].

The use of molecular tools has become essential to understand the source, transmission routes, and virulence of *Klebsiella pneumoniae* infections. In recent years, infections due to hypervirulent *K. pneumoniae* isolates have been a significant cause of morbidity and mortality. Multi-drug resistant hypervirulent isolates can harbor mosaic plasmids that carry both antibiotic resistance genes and virulence genes. In previous studies, ST23, ST11, ST15, and ST147 were reported as hypervirulent clones [11]. In 2022, carbapenem resistant ST 2096 isolates with mosaic plasmids carrying antimicrobial resistance genes (aadA2, armA, blaOXA-1, msrE, mphE, sul1 and dfrA14) and virulence genes (rmpA2, iutA and iucABCD) were detected in a whole genome sequencing (WGS) study. It was reported that the mosaic plasmid carries its own type IV-A3 CRISPR-cas system that can target the acquisition of the IncF plasmid with the aid of a traLspacer [11]. In a 2019 Saudi Arabia study conducted on 235 *K. pneumoniae* isolates, ST2096 and ST14 were reported as the dominant highly virulent sequence types and classified in CC14 [12]. In Italy, ST512, ST101, ST307 were common among *K. pneumoniae* isolates and ST307 and ST101 showed multidrug antibiotic resistance [13]. In a recent study conducted in Romania, ST101 *K. pneumoniae* isolates were detected in samples collected from hospitalized patients and water samples taken from the inlets and outlets of a hospital sewage tank [14]. In a 2021 study in Turkey, seven STs including ST14, ST16, ST79, ST101, ST1543, ST2096, and ST2832 were identified by MLST. ST14 (81%) and ST2096 (94%) classified in CC14 were the most common STs [15]. In our study, ST 2096 was the most common. The remaining STs were ST101, ST 147, ST 15, ST16, ST391, ST3681, ST345, ST336, ST14, ST22, ST307, ST37, and ST985. In İstanbul and Ankara ST2096, in Sivas ST15, in Diyarbakır ST391 and ST101, in İzmir ST14, in Isparta ST101 were the most common ST types. The clonal complex 14 is common in Istanbul, Ankara, and Izmir, which have the highest patient circulation from different provinces of Turkey. This data demonstrated the necessity of monitoring hypervirulent ST2096 CC14 *K. pneumoniae* isolates in Turkey.

In conclusion, carbapenem and/or colistin-resistant *K. pneumoniae* is spreading rapidly in hospitals in Turkey. A cross-transmission of ST2096 CC14 *K. pneumoniae* was detected in six Geographic regions of Turkey. Infection control measures include awareness and education of healthcare workers, ensuring contact isolation in patients, daily chlorhexidine bathing of patients, limitation of invasive device use, shortening the mechanical ventilation duration, giving importance to hand hygiene, management of antimicrobial use, and increasing environmental cleanliness are necessary. The use of molecular typing tools will highlight an understanding of the transmission dynamics of resistant *K. pneumoniae* within the hospitals. Large-scale studies investigating the distribution and evolution of hypervirulent isolates are required.

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