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Original Article

Investigation of colistin heteroresistance and the colistin resistance genes *mcr*-1 to *mcr*-5 in *Escherichia coli* and *Klebsiella pneumoniae* isolates in a tertiary hospital in Turkey

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Abstract

Introduction: Heteroresistance is not detected by traditional antimicrobial susceptibility testing methods and may lead to treatment failures. Investigating the presence of plasmid-mediated colistin resistance genes is important because of the horizontal transmission of the relevant genes between bacterial species. This study aimed to investigate the presence of colistin heteroresistance and the colistin resistance genes *mcr*-1 to *mcr*-5 in *Escherichia coli* and *Klebsiella pneumoniae* isolates.

Methodology: A total of 254 isolates, including 100 *E. coli* and 154 *K. pneumoniae* strains isolated from clinical samples, were included in the study. Colistin susceptibility was evaluated using the broth microdilution method for all strains. Heteroresistance screening was performed using the gradient strip test. Eight strains were evaluated for heteroresistance by population analysis profiling (PAP). The colistin resistance genes *mcr*-1 to *mcr*-5 were investigated by multiplex polymerase chain reaction (PCR) in colistin-resistant *K. pneumoniae* isolates. Multilocus sequence typing (MLST) analysis was performed on two *K. pneumoniae* strains.

Results: Colistin resistance was not detected in the *E. coli* isolates and was detected in 16.23% (25/154) of the *K. pneumoniae* isolates. No heteroresistant bacteria were detected by the gradient strip test or by PAP. All colistin-resistant isolates were negative for the *mcr* genes. The two isolates analyzed by MLST were ST14 and ST2096.

Conclusions: Periodic follow-up of colistin heteroresistance is useful for administering appropriate antibiotic therapy. In addition, the investigation of colistin resistance genes is important for infection control measures.

Key words: Colistin; heteroresistance; *mcr* genes.

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Introduction

Colistin is a cationic polypeptide antibiotic belonging to the class of polymyxins that was first used clinically in the late 1950s. Polymyxins have often been used to treat infections caused by Gram-negative bacteria resistant to other antibiotics. However, the use of colistin has declined due to serious systemic side effects, such as nephrotoxicity and neurotoxicity, and the availability of less toxic antibiotics that can be used for treatment [1]. After the mid-1990s, the limited treatment options for bacteria with multiple drug resistance, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae,* led to increased interest in colistin [2].

Heteroresistance can be defined as the presence of heterogeneous bacterial populations in which one or more subpopulations exhibit increased levels of antibiotic resistance compared to the main population. In heteroresistance, different responses to a particular antibiotic are observed in seemingly identical bacterial cells [3,4]. Since heteroresistance can lead to treatment failure, it is important to detect this phenomenon for appropriate antibiotic selection [4,5].

Although heteroresistance has been demonstrated in many antibiotics and bacteria, few epidemiological data on its prevalence exist [3,6]. Some studies have revealed colistin heteroresistance in bacterial species such as *Enterobacter cloacae* complex [7,8], *A. baumannii* [9,10], *K. pneumoniae* [11,12], *Stenotrophomonas maltophilia* [13], and *Escherichia coli* [11,14,15]. Colistin is a difficult drug to use due to its physicochemical properties and low therapeutic index [16,17]. In addition, heteroresistance to this antibiotic, which is preferred for multidrug-resistant bacterial infections, may lead to treatment failures [6].

The *mcr* genes encode MCR proteins, which are cytoplasmic transmembrane proteins found in Gram-negative bacteria. These proteins have a phosphoethanolamine (pEtN) transferase function, attach a pEtN moiety to the lipid A of the

lipopolysaccharide in the bacterial cell membrane, and remove the negative charge of lipid A. As a result, the affinity of cationic polymyxins to lipid A decreases, leading to antibiotic resistance [18]. By January 2022, 10 mobile colistin resistance genes, *mcr*-1 to *mcr*-10, had been described within eleven Enterobacteriaceae species [19,20]. In all continents except Antarctica, *mcr* gene variants have been found in isolates obtained from humans, animals, and the environment [21].

Multilocus sequence typing (MLST) is a nucleotide sequence-based method developed to characterize the genetic relationships among bacterial isolates. This method provides data that can be compared in international databases [22].

In this study, we aimed to investigate the rate of colistin resistance and of heteroresistance frequency in *E. coli* and *K. pneumoniae* isolates and the presence of the *mcr*-1 to *mcr*-5 genes in colistin-resistant ones of those isolates. Additionally, we aimed to perform MLST analysis on the colistin-resistant isolates with a high minimum inhibitory concentration (MIC).

Methodology

This study was approved by the Ethical Committee of the Trakya University School of Medicine (TUTF-BAEK 2020/406).

Bacterial isolates and identification

A total of 254 *E. coli* and *K. pneumoniae* isolates obtained from hospitalized patients at the Trakya University Health Center for Medical Research and Practice between November 2020 and May 2021 were included in the study. Each isolate represented a single sample from one patient or a different type of sample (such as blood, urine, sputum) from the same patient. The *E. coli* and *K. pneumoniae* isolates were stored at -80 °C for the analysis. The bacterial isolates were identified by conventional methods and the VITEK 2® compact microbial identification system using VITEK 2® GN identification cards (bioMérieux, France, lot no. 2411515203) according to the manufacturer's recommendations. For identification by conventional method, the oxidase testing was carried out to the isolates. Oxidase-negative isolates were tested on a group of media where their biochemical properties were evaluated. These media were TSI (triple sugar iron agar), LIA (lysine iron agar), MIO (motility, indole, ornithine), FAD (phenylalanine deaminase), Simmons' citrate agar, and urea agar media.

Antimicrobial susceptibility testing

The broth microdilution method was performed in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) v13 recommendations to determine the colistin susceptibility of the isolates. Colistin-resistant *E. coli* (NCTC 13846) and colistin-susceptible *E. coli* (ATCC 25922) were used as control strains. The *E. coli* and *K. pneumoniae* isolates with MIC > 2 μg/mL were considered resistant, and the isolates with MIC ≤ 2 μg/mL were considered susceptible. The results of susceptibility testing to the carbapenem antibiotics (imipenem, meropenem, and ertapenem) determined by the disk diffusion method and the extended-spectrum beta-lactamase (ESBL) determined by the double-disk synergy test were examined retrospectively from laboratory records [23].

Heteroresistance screening and population analysis profiling (PAP)

The bacterial isolates were screened for the presence of heteroresistant subpopulations using the gradient strip test method [3]. Colistin MIC test strips (0.016–256 mg/L, lot no. 111220049, Liofilchem, Italy) were used in the heteroresistance screening test. PAP was performed on eight isolates (five *E. coli* and three *K. pneumoniae*) that were positive for ESBL and/or resistant to carbapenems [24]. Briefly, Mueller Hinton agar (MHA) plates containing colistin at concentrations of 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 mg/L were prepared. 10-fold serial dilutions were made from the bacterial suspension at a concentration of 10^8 CFU/mL and 50 μL from each dilution was spread onto each MHA agar plate containing colistin. The plates were incubated at 35-37 °C for 48 hours. The number of colonies grown on each plate was recorded and a semilogarithmic graph was obtained.

Screening for the presence of the mcr-1 to mcr-5 genes

A PureLink Genomic DNA Mini Kit (Invitrogen, USA, lot no. 1874869) was used to isolate the DNA. The multiplex PCR protocol previously described by Rebelo *et al.* was used to detect the *mcr*-1 to *mcr*-5 genes [25]. PCR products were subjected to electrophoresis on 2% agarose gel, and the bands were evaluated under ultraviolet light. *mcr*-1-positive *E. coli* (NCTC 13846) was used as a positive control, and *E. coli* (ATCC 25922) was used as a negative control. The primers used in this study are shown in Table 1 [25,26].

Table 1. Primers used for multiplex PCR for detection of *mcr*-1 to *mcr*-5 genes.

Target Gene	Oligonucleotide primers	Amplicon size (bp)	Reference
mcr-1	Forward: 5'/ - AGTCCGTTTGTTCTTGTGGC -/3'	320	25
	Reverse: 5'/ - AGATCCTTGGTCTCGGCTTG -/3'		
mcr-2	Forward: 5'/ - CAAGTGTGTTGGTCGCAGTT -/3'	715	25
	Reverse: 5'/ - TCTAGCCCGACAAGCATACC -/3'		
mcr-3	Forward: 5'/ - AAATAAAAATTGTTCCGCTTATG -/3'	929	25
	Reverse: 5'/ - AATGGAGATCCCCGTTTTT -/3'		
mcr-4	Forward: 5'/ - TCACTTTCATCACTGCGTTG -/3'	1116	25
	Reverse: 5'/ - TTGGTCCATGACTACCAATG -/3'		
mcr-5	Forward: 5'/ - ATGCGGTTGTCTGCATTTATC -/3'	1644	26
	Reverse: 5'/ - TCATTGTGGTTGTCCTTTTCTG -/3'		

Multi-locus sequence typing

First, seven gene regions of the isolates (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB,* and *tonB*) were PCR amplified. Then, PCR for sequencing was carried out, followed by sequencing [22]. Sequence analysis of the PCR products was carried out on an Applied Biosystems 3730XL DNA Analyzer with an Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA, USA). The sequencing chromatograms were analyzed using the ProSeq v2 and BioEdit programs, and the nucleotide sequences of the isolates were uploaded to the MLST database [27].

Results

Bacterial isolates and identification

Of the 254 bacterial strains, 154 (60.63%) were *K. pneumoniae*, and 100 (39.37%) were *E. coli*. The distribution of the isolated bacteria by sample type is shown in Table 2.

Antimicrobial susceptibility testing

Of the 254 bacterial strains, 25 (9.84%) were found to be colistin-resistant, and 229 (90.16%) were found to be susceptible. While no resistance to colistin was detected in any of the *E. coli* isolates, colistin resistance was observed in 25 *K. pneumoniae* isolates (16.23%). The MIC value of the four resistant isolates was 8 mg/L, and the MIC value of the other four isolates was 16 mg/L. Fifteen isolates had a MIC of 32 mg/L, and two isolates had a MIC of 64 mg/L.

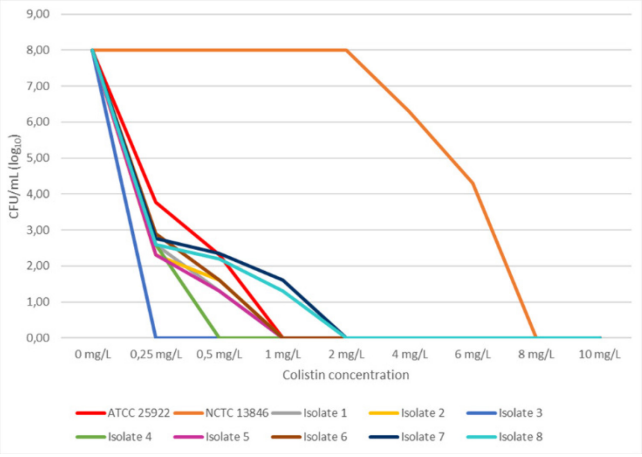
Heteroresistance screening and PAP

No resistant subpopulations were found in the inhibition zone on Mueller Hinton agar in the bacterial isolates screened using the gradient strip test method. No heteroresistant bacteria were detected as a result of the PAP performed on eight strains that were ESBL positive and/or resistant to carbapenems. The population analysis profile of the control strains and clinical isolates is shown in Figure 1.

Screening for the presence of the mcr-1 to mcr-5 genes

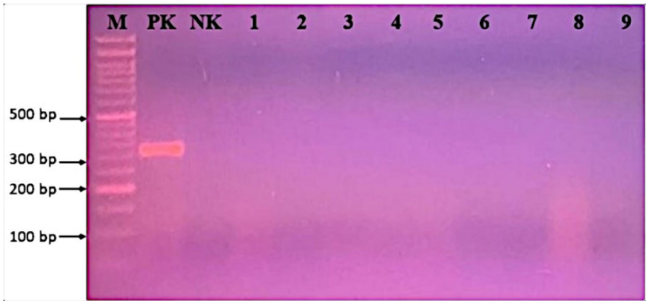
All 25 colistin-resistant *K. pneumoniae* isolates were found to be negative for the *mcr*-1 to *mcr*-5 genes. Representative gel of *mcr*-1 to *mcr*-5 genes investigated from colistin-resistant *K. pneumoniae* isolates is shown in Figure 2.

Figure 1. Population analysis profile of the control strains and clinical isolates.



CFU: Colony forming unit.

Figure 2. Representative gel of *mcr*-1 to *mcr*-5 genes investigated from Klebsiella pneumoniae isolates.



M; DNA marker, PK; positive control, NK; negative control, lane 1-9; PCR products of the samples.

Table 2. Distribution of isolated bacteria by sample type.

Sample type	*E. coli*	*K. pneumoniae*	Total (%)
Blood	21	57	78 (30.70)
Urine	45	32	77 (30.31)
Sputum	3	35	38 (14.96)
Aspiration material	13	11	24 (9.45)
Tissue biopsy	11	7	18 (7.09)
Catheter	4	8	12 (4.72)
Wound swab	2	1	3 (1.18)
Pleural fluid	0	2	2 (0.79)
Cerebrospinal fluid	0	1	1 (0.39)
Peritoneal fluid	1	0	1 (0.39)
Total	100	154	254 (100)

Multi-locus sequence typing

MLST analysis was performed on two *K. pneumoniae* isolates (isolates 24 and 33), whose colistin MIC was 64 mg/L. Isolate 24 was determined as ST2096 and isolate 33 was determined as ST14. The allelic profiles and sequence types of the isolates are shown in Table 3.

Discussion

Antimicrobial resistance (AMR) is a significant concern for public health worldwide. Increased AMR causes difficulties in the treatment of bacterial infections, therapeutic complications, prolonged hospital stays, and increased mortality [28]. Antimicrobial-resistant bacteria cause more than 35,000 deaths each year in European Union/ European Economic Area (EU/EEA) countries [29]. Colistin is used for infections caused by multidrug-resistant Gram-negative bacteria, especially carbapenemase-producing Enterobacterales, *P. aeruginosa*, and *A. baumannii* [30].

According to Antimicrobial Testing Leadership and Surveillance (ATLAS) data, the colistin resistance rate was 4.73% (323/6826) in *K. pneumoniae* and 0.45% (32/7074) in *E. coli* worldwide in 2021. In European countries, it was 7.62% (173/2271) in *K. pneumoniae* and 0.54% (13/2387) in *E. coli*. In the same year, the colistin resistance rate in Turkey was 29.34% (49/167) in *K. pneumoniae* and 0.57% (1/175) in *E. coli*. Between 2017 and 2021, the rate of colistin resistance in Turkey was indicated as 22.57% (151/669) in *K. pneumoniae* and 0.16% (1/625) in *E. coli* [31]. In this study, while colistin resistance was not detected in the *E. coli* isolates, resistance was found in 25 (16.23%) of the 154 *K. pneumoniae* strains. Süzük Yıldız *et al*. found colistin resistance to be 8.7% in *E. coli* and 28.4% in *K. pneumoniae* in isolates collected from 26 hospitals in Turkey [32]. The limited number of isolates in our study and the possibility of differences in the resistance profiles of each hospital may explain the different rates between studies. However, taking into account the results of our study and other studies conducted in our country, it is seen that colistin resistance is at a considerable level in both these bacterial species, especially in *K. pneumoniae* [32-34].

Table 3. Allelic profile and sequence types of isolates.

Gene	Allelic profile, isolate 24	Allelic profile, isolate 33
infB	6	6
mdh	1	1
rpoB	46	1
tonB	1	1
gapA	1	1
pgi	1	1
phoE	1	1
Sequence type	ST2096	ST14

Heteroresistance is a form of resistance in which a strain has a population to which the majority of cells are susceptible, along with a resistant subpopulation. Heteroresistance may not be detected by conventional clinical laboratory testing methods. Heteroresistant isolates may lead to incorrect results in antibiotic susceptibility categorization, which may lead to inappropriate therapy and treatment failure [35,36]. Juhasz *et al*. found heteroresistance at a rate of 3.4% in *E. coli* and 48.6% in *K. pneumoniae* isolates obtained from blood culture at a tertiary care center in Hungary [14]. The heteroresistance rate detected by PAP in carbapenem-resistant Enterobacterales isolates collected in the USA between 2012 and 2015 was found to be 10.1% [36]. In a study conducted in Egypt, 23.3% heteroresistance was found in colistin-susceptible *K. pneumoniae* isolates [37]. In this study, no heteroresistant bacteria were detected in the screening of the colistin-susceptible strains using the gradient strip test method. Thereupon, PAP was performed on eight bacterial strains, of which five were *E. coli* and three were *K. pneumoniae*, positive for ESBL and/or carbapenem resistance, and no heteroresistant strains were detected. These results differ from the prevalence found in other studies on colistin heteroresistance, which may relate to the characteristics of the study populations.

Until the plasmid-mediated colistin resistance (*mcr*-1) gene was described by Liu *et al*., colistin resistance was thought to be associated only with mutational and regulatory changes mediated by chromosomal genes [38]. The spread of plasmid-mediated genes and antimicrobial resistance by horizontal transfer poses a risk for colistin, which is considered a last-line treatment for infections caused by multidrug-resistant Gram-negative bacilli [39]. In a study conducted in Russia that included 4324 *E. coli* and 4530 *K. pneumoniae* strains, the *mcr*-1 gene was found at a rate

of 0.51% in the *E. coli* strains and 0.04% in the *K. pneumoniae* strains [40]. Falgenhauer *et al*. investigated the *mcr*-1 gene in 577 Gram-negative bacteria that were ESBL- and carbapenemase-producing and obtained from humans, animals, and the environment. Of the four *E. coli* isolates positive for the *mcr*-1 gene, three were obtained from pigs and one from a human wound swab. While *mcr*-1 positivity was 0.45% of the 223 *E. coli* isolates obtained from humans, all 29 *K. pneumoniae* isolates were negative [41]. Moubareck *et al*. investigated *mcr*-1, *mcr*-2, *mcr*-3, and *mcr*-4 genes in carbapenem- and colistin-resistant *K. pneumoniae* strains obtained from clinical samples, and no *mcr* genes were detected [42]. Hameed *et al*. detected the *mcr*-3 gene in one of 127 *E. coli* clinical isolates in China [43]. Sarı *et al*. investigated the *mcr*-1 and *mcr*-2 genes in 329 clinical Enterobactarales isolates, most of which were *K. pneumoniae* strains, in a multicenter study conducted in Turkey and found all strains to be negative [44]. Isler *et al.* did not detect the *mcr-1* gene in carbapenem-resistant *K. pneumoniae* strains isolated from patients with bloodstream infections in Turkey [45]. In another study conducted in Turkey, the *mcr*-1 gene was investigated in 57 carbapenem-resistant *K. pneumoniae* strains, and three isolates were found to be positive [46]. In this study, we investigated the presence of the *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, and *mcr*-5 genes in 25 colistin-resistant *K. pneumoniae* strains, and we did not detect *mcr* genes in any of the strains. In our study, similar results were found to the studies conducted in Turkey including clinical isolates. As our study and other studies have shown, the prevalence of *mcr* genes in clinical isolates in Turkey is low.

MLST is a DNA sequence-based method that can be used to investigate the molecular characterization and genetic relationships of many bacterial strains [47]. In this study, one of the two *K. pneumoniae* isolates analyzed by MLST was ST2096, and the other one was ST14. ST14 and ST2096 are both located in clonal complex 14, and ST2096 is a single locus variant of ST14 [48]. *K. pneumoniae* ST14 has been reported in some Asian countries (India, China, Singapore, Israel, and Pakistan), Turkey, and Australia [49]. ST14 is one of the most common causes of nosocomial infections in many countries, and its plasmids encode many β-lactamases [50]. *K. pneumoniae* ST2096 has been reported in Saudi Arabia, India, Pakistan, and Turkey [45,48,51-53]. Isler *et al*. examined carbapenem-resistant *K. pneumoniae* strains in a multicenter study in Turkey. It was determined that 61 (32.6%) of 187 isolates were ST2096, 37 (19.8%) were ST101, and 28 (15.0%) were ST14 clones. Colistin resistance rates in the ST2096, ST101, and ST14 strains were 18%, 14%, and 36%, respectively [45]. Since MLST analysis was performed on a limited number of isolates in our study, a sufficient comparison of results with other studies could not be made. However, the determination of the sequence types of these two strains with high colistin MIC values as ST14 and ST2096 seems to be compatible with the literature data in that these two types are among the sequence types that frequently cause nosocomial infections and may be resistant to colistin [45,48,50]. In addition, according to the antibiotic susceptibility test results obtained from routine laboratory processes for these two strains for which MLST was performed, while ST2096 was resistant to penicillins, cephalosporins, carbapenems other than imipenem, amikacin, ciprofloxacin, aztreonam, and trimethoprim/sulfamethoxazole, ST14 was resistant to penicillins, cephalosporins, carbapenems, amikacin, and ciprofloxacin.

Conclusions

This study showed that colistin resistance was high in *K. pneumoniae* strains and low in *E. coli* strains in our center, which is a tertiary university hospital serving the Thrace region. There was no heteroresistance in these two bacterial species, and colistin-resistant *K. pneumoniae* strains did not have the *mcr*-1 to *mcr*-5 gene. Since heteroresistance is a condition that may not be detected by traditional antimicrobial susceptibility tests, determining its prevalence will guide clinical practice and the planning of laboratory diagnostic methods. There is no cause for concern in terms of heteroresistance in our region for now; however, we think that heteroresistance should be screened periodically. Investigation of plasmid-mediated colistin resistance genes is important because these genes are transferred between bacterial species by horizontal transmission. In addition, this study showed that the two *K. pneumoniae* strains with high colistin MIC values are ST14 and ST2096 clones, where resistance to colistin as well as other antibiotics is frequently observed. The bacterial sequence type determined by MLST analysis, although limited due to the low number of isolates in this study, can be compared with other molecular epidemiological studies.

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