Clin. Lab. 2021;67:XXX-XXX

ORIGINAL ARTICLE

Carbapenem and Colistin Resistant *Klebsiella Pneumoniae* ST14 and ST2096 Dominated in Two Hospitals in Turkey

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SUMMARY

Background: **The determination of clonal interactions between microorganisms is very important in epidemiological studies. In the present study, we aimed to evaluate the resistance mechanisms and genetic relationships of carbapenem and colistin resistant *Klebsiella pneumoniae* (*K. pneumoniae*) strains isolated from inpatients at two university hospitals in Turkey.**
Methods: **A total of 38 *K. pneumoniae* clinical isolates were included in the study. Identification of isolates was confirmed by 16S rRNA sequencing. Antimicrobial susceptibility testing was performed with VITEK-2 system (bioMérieux, France). Modified Hodge test was used for the detection of carbapenemase activity in isolates. Carbapenem resistance genes (*bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$) and colistin resistance genes (*mcr-1*, *mcr-2* and *mcr-3*) were investigated by PCR. Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and multilocus sequence typing (MLST) analysis were used to determine the genetic relatedness among the isolates.**
Results: **We detected that 58% of isolates were positive for only *bla*$_{OXA-48}$, 5% were only positive for *bla*$_{NDM}$, and 34% were positive for both *bla*$_{OXA-48}$ and *bla*$_{NDM}$. *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *mcr-1*, *mcr-2*, and *mcr-3* were not detected among the isolates. Only one carbapenem resistant isolate was negative for the carbapenemase genes tested. A total of nine profiles were found by ERIC-PCR, and MLST results showed seven different sequence types-ST14, ST16, ST79, ST101, ST1543, ST2096, and ST2832. The seven STs were grouped by *PHYLOVIZ Online* into four clonal complexes. The most common ST was ST14 (81%) in Center 1 and ST2096 (94%) in Center 2.**
Conclusions: **We determined MLST types of carbapenem and colistin resistant *K. pneumoniae* isolates from two different centers. Although the most common ST types were different in these centers, both ST types were clustered in CC14. To the best of our knowledge this is the first report of ST14 and ST2096 outbreaks in Turkey.**
(Clin. Lab. 2021;67:xx-xx. DOI: 10.7754/Clin.Lab.2021.201226)

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Manuscript accepted February 10, 2021

KEY WORDS

Klebsiella pneumoniae, colistin, carbapenemase, multilocus sequence typing

INTRODUCTION

Antibiotic resistance is gradually spreading and causing treatment failures. The strains which are currently resistant to all common antibiotics are primarily isolated from Enterobacterales especially from *Klebsiella* spp.

Klebsiella pneumoniae (*K. pneumoniae*) is a major cause of hospital acquired infections such as lower respiratory tract infections, bloodstream infections, septicemia, and infections in intensive care unit patients. In some countries, due to multiple antimicrobial resistance mechanisms, even carbapenems are not effective in more than half of the patients treated for *K. pneumoniae* infections [1].

Some antimicrobials retain activity against carbapenem-resistant *K. pneumoniae*. These generally include aminoglycosides, tigecycline, and ceftazidime/avibactam. Additionally, polymyxins including colistin are an important treatment option. Colistin resistance is most commonly detected in *Escherichia coli* (*E. coli*), but is present in several genera, including *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, and *Enterobacter* [2]. These cationic antimicrobial peptides have emerged as a "last line" therapeutic option for various multiple drug resistant organisms [3].

The determination of clonal interactions of microorganisms is very important in epidemiological studies. Pathogenesis studies and bacterial genetics are important contributions to many research areas such as molecular typing of microorganisms, epidemiological surveillance of infectious diseases, evaluation of outbreaks, and prognosis of diseases. In this study, resistance mechanisms and genetic relationships of carbapenem and colistin resistant *K. pneumoniae* strains obtained from inpatients in various clinical, intensive care and wound care units in City 1 university hospital and City 2 university hospital between 2017 and 2018 were investigated. The study was conducted after sample collection was completed.

MATERIALS AND METHODS

Isolates and identification tests

A total of 38 (21 from City 1, and 17 from City 2) non-duplicate *K. pneumoniae* isolates were collected from inpatients in various clinics and intensive care units at two university hospitals in Turkey (City 1 in the western part, and City 2 in the central part). The isolates were checked for purity and colony morphology specific to *Klebsiella* species and identified by VITEK-2 system (bio-Mérieux, France) in City 1 and MALDI-TOF MS (Bruker, Germany) in City 2.

The identification of all strains was verified by 16S rRNA sequencing analysis. Universal 16S rRNA bacterial primers 20F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1390R (5'-GAC GGG CGG TGT GTA CAA-3') were used to amplify the gene using 10 ng of genomic DNA isolated from each strain. PCR products were visualized on a 1% agarose gel stained with safe view under UV light to confirm the presence of a ~1,350 bp PCR products. Amplicons were purified and directional sequenced with primer 20F to verify the identifications.

Antimicrobial susceptibility testing

The Vitek 2 (bio-Mérieux, France) system was used for antimicrobial susceptibility testing (AST) of *Klebsiella* species according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) criteria. The antimicrobial agents tested were amikacin, ampicillin, colistin, cefazolin, cefoxitin, ceftazidime, ciprofloxacin, ertapenem, gentamicin, imipenem, meropenem, piperacillin-tazobactam, ceftriaxone, tigecycline, and trimethoprim-sulfamethoxazole.

Detecting carbapenemase production

The isolates were tested for carbapenemase production by the Modified Hodge Test (MHT). *E. coli* ATCC 25922 usually grows better within the carbapenem disc inhibition zones with carbapenemase producers. For this reason, production of carbapenemase was discriminated by looking at the growth pattern of carbapenem susceptible *E. coli* around the tested isolate streak zones within the carbapenem disc inhibition area on MHT [4].

DNA extraction for PCR amplification

All isolates were inoculated from a single colony and incubated aerobically in tryptic soy broth for 18 - 24 hours at 37°C. DNA extraction was performed by using InstaGene Matrix DNA extraction kit (Bio-Rad) according to the manufacturer's recommendations.

Detection of carbapenem and colistin resistance genes by PCR

For detection of the genes, bla_{OXA-48}, bla_{NDM}, bla_{KPC}, bla_{IMP}, bla_{VIM}, *mcr-1*, *mcr-2*, *and mcr-3* specific primer pairs were used for each gene listed in Table 1. PCR amplifications were performed in 50 µL reaction mixtures containing 1 µL of dNTPs (10 mM), 0.4 µL of each primer (100 pmol), 5 µL Taq buffer with Mg+ (10 X), 0.6 µL Taq DNA polymerase (5 U/µL) (ABM, Canada), and template DNA. Each gene was amplified separately. The resistance genes were amplified by using the following protocol: held at 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (50 - 60°C for 30 seconds) and extension (72°C for 1 minute), with a single final extension of 7 minutes at 72°C. PCR products were analyzed after electrophoresis in 1% agarose gel stained with Safe View (ABM, Canada) for 30 minutes at 100 V. PCR fragments were visualized by UV light and identified by their size shown in Table 1 [5-8].

Enterobacterial repetitive intergenic consensus (ERIC)-PCR and multilocus sequence typing (MLST)

Enterobacterial repetitive intergenic consensus (ERIC)-PCR was applied to examine the genetic relatedness of all collected isolates. One primer sequence was used in this amplification (ERIC2:
5′-AAGTAAGTGACTGGGGTGAGCG-3′) [9].

The amplification conditions were initial denaturation at

Table 1. The primer sequences used in PCR for detecting carbapenemase and colistin resistance genes in *K. pneumoniae* strains.

Gene	Primer sequences (5′ - 3′)	Product size (bp)	Reference
bla_{OXA-48}	TGTTTTTGGTGGCATCGAT	177	[5]
	GTAAMRATGCTTGGTTCGC		
bla_{NDM-1}	TTGGCCTTGCTGTCCTTG	82	[5]
	ACACCAGTGACAATATCACCG		
bla_{KPC}	TCGCTAAACTCGAACAGG	798	[5]
	TTACTGCCCGTTGACGCCCAATCC		
bla_{IMP}	GAGTGGCTTAATTCTCRATC	120	[5]
	AACTAYCCAATAYRTAAC		
bla_{VIM}	GTTTGGTCGCATATCGCAAC	382	[5]
	AATGCGCAGCACCAGGATAG		
mcr-1	CGGTCAGTCCGTTTGTTC	309	[6]
	CTTGGTCGGTCTGTAGGG		
mcr-2	TGTTGCTTGTGCCGATTGGA	567	[7]
	AGATGGTATTGTTGGTTGCTG		
mcr-3	TTGGCACTGTATTTTGCATTT	542	[8]
	TTAACGAAATTGGCTGGAACA		

Table 2. Allelic profiles and sequence types (STs) and ERIC-PCR profiles assigned in the *K. pneumoniae* MLST database for the strains from Turkey.

ST Type (Clonal Complex)	Allel profiles							ERIC-PCR profiles	Number of isolates
	gapA	infB	mdh	pgi	phoE	rpoB	tonB		
ST79 (14)	1	6	5	1	1	5	1	A	1
ST14 (14)	1	6	1	1	1	1	1	B, E, G	17
ST16 (16)	4	2	2	1	4	1	4	F	1
ST1543 (15)	1	21	141	1	1	85	1	C	1
ST101 (101)	2	6	1	5	4	1	6	D	1
ST2096 (14)	1	6	1	1	1	46	1	H	16
ST2832 (15)	1	1	1	1	1	1	401	J	1

94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 42°C for 30 seconds, and 72°C for 2 minutes, and a final elongation at 72°C for 7 minutes. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels stained with safe dye. ERIC-PCR band patterns were determined using ERIC2 primers and gel electrophoresis images were used to assign clonal relations of the isolates. The same gel patterns were accepted as the same clones. All different band patterns are accepted as different clones.

Genotyping of the distinct ERIC-PCR clones was performed by MLST comparing seven housekeeping genes (*phoE, gapA, rpoB, tonB, inf, mdh, and pgi*) according to the protocol published on the Institute of Pasteur website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

To conclude the allele type of each gene, sequences were compared to already defined alleles. For each isolate, allele types of seven genes were detected, and then these allele types were used to determine the sequence type. STs of clones were determined using the same website online tool. The program PHYLOVIZ Online was used to identify the clonal complexes (CC) (https://online.phyloviz.net/index). Data from additional 3483 *K. pneumoniae* isolates were obtained from the MLST isolate database deposited at the Pasteur Institute [10].

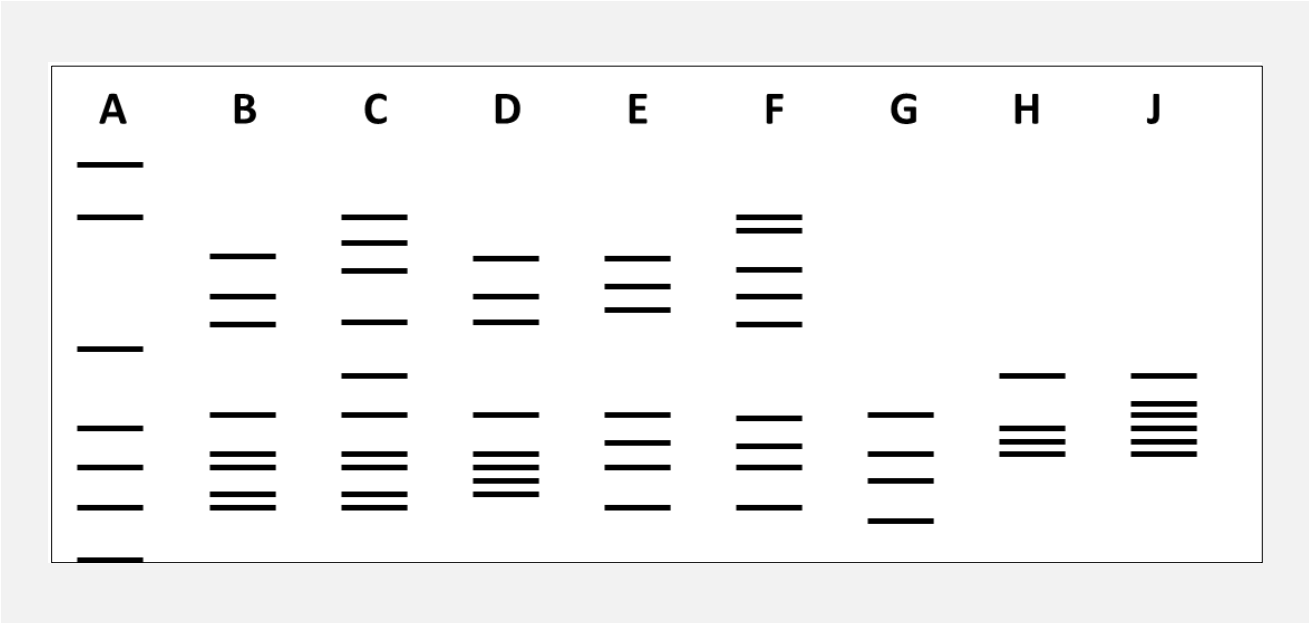

Figure 1. ***K. pneumoniae*** **ERIC-PCR patterns detected in the study.**

A total of nine profiles were detected among isolates. ERIC-PCR patterns B, E, and G were all found to be ST14, A was ST79, C: ST1543, D: ST101, H: ST2096, and J: ST2832.

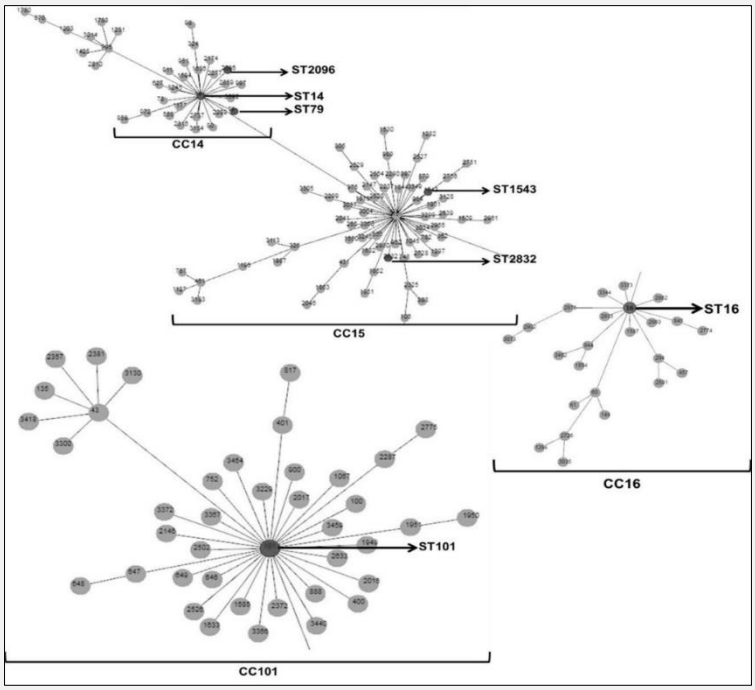

Figure 2. ***K. pneumoniae*** **STs and eBURST clonal complex identified in the study. eBURST snapshot of** ***K. pneumoniae*** **and closely related species.**

Dark dots represent individual MLST sequence types from hospitals indicated by arrows.

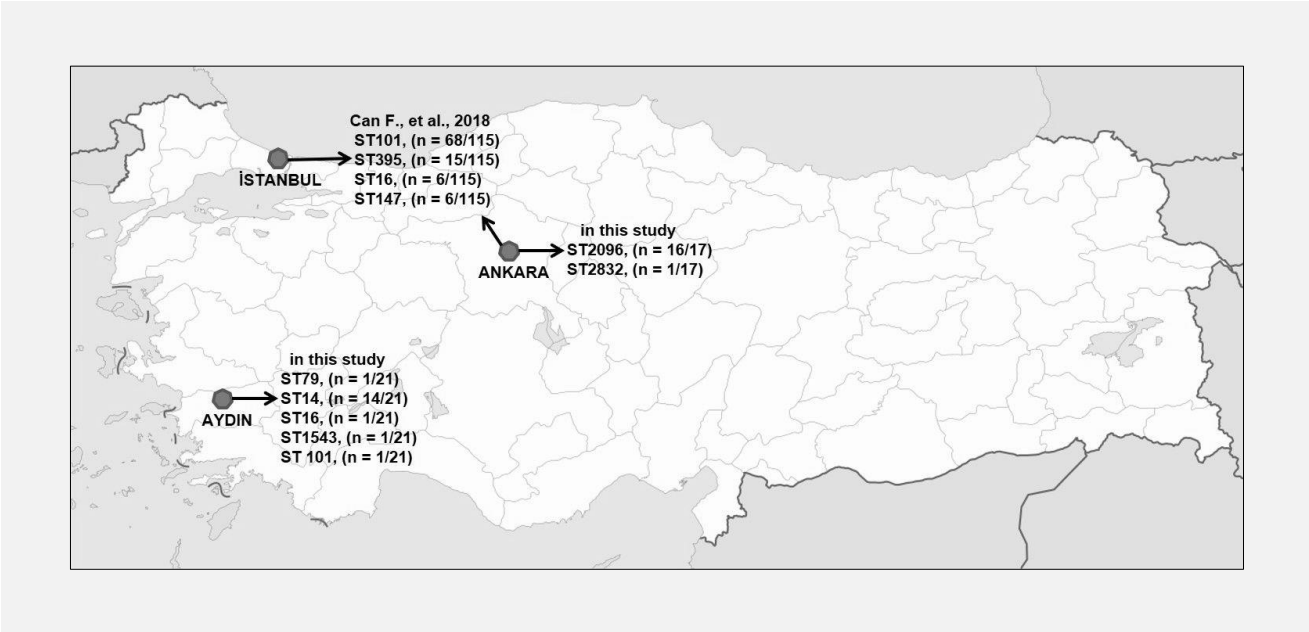

Figure 3. Geographical distribution of ST types for clinical isolates of *K. pneumoniae* from Turkish hospitals.

RESULTS

Between November 2017 and September 2018, a total of 38 *K. pneumoniae* isolates from two hospitals were collected. Among these isolates, 19 were from internal medicine intensive care unit, 12 were from anesthesia and reanimation intensive care unit, three were from coronary intensive care unit, two were from hematology unit, one was from general medicine intensive care unit, and one was from neurology intensive care unit. Isolates were obtained from blood (n = 13), trans-tracheal aspirates (n = 13), urine (n = 9), sputum (n = 1), bronchoalveolar lavage (n = 1), and peripheral venous catheter (n = 1) samples.

Carbapenemase activity was found positive with the Modified Hodge Test in all 38 isolates. All strains were evaluated for detection of carbapenem and colistin resistance genes by PCR. Of the 38 isolates, 35 (92%) were bla_{OXA-48} and 15 (39%) were bla_{NDM} positive. Of 38 isolates, 22 (58%) were positive for only bla_{OXA-48}, 2 (5%) were only positive for bla_{NDM}, and 13 (34%) were positive for both bla_{OXA-48} and bla_{NDM} genes. Other resistance genes such as bla_{KPC}, bla_{IMP}, bla_{VIM}, and *mcr-1*, *mcr-2*, *mcr-3* were negative.

A total of nine profiles were found by ERIC-PCR. These nine clones from ERIC-PCR fingerprinting profiles are shown in Figure 1.

Seven different sequence types (STs) were identified by MLST with nine different patterns obtained by ERIC-PCR. Five of the STs contained single isolates, while two STs included 17 and 16 isolates. Allelic profiles, sequence types (STs) and ERIC-PCR profiles detected in the *Klebsiella* MLST database of strains are shown in Table 2.

The seven STs were separated by *PHYLOVIZ Online* into four clonal complexes by sharing alleles at six of seven loci (Figure 2). The most dominant ST in City 1 university hospital (in the west part of Turkey) was ST14 (80.9 %, 17/21), followed by ST16 (4.7%, 1/21), ST79 (4.7%, 1/21), ST101 (4.7%, 1/21), and ST1543 (4.7%, 1/21). The most dominant ST in City 2 university hospital (in central Turkey) was ST2096 (94%, 16/17), followed by ST2832 (6%, 1/17). The most common ST was ST14 (81%) in City 1 and ST2096 (94%) in City 2. Clones of both hospitals were found as ST14, ST16, ST79, ST101, ST1543, ST2096, and ST2832. Those seven STs accounted for 18% (7/38) of the total isolates, and those seven isolates were therefore designated prevalent clones in this study.

DISCUSSION

The present study describes the genetic diversity of colistin and carbapenem resistant *K. pneumoniae* isolates in two different training and research hospitals in western (City 1) and central (City 2) Turkey. Similar to the results found in a large multicenter study conducted in Turkey, bla_{OXA-48} showed a high frequency in both City 1 and City 2 for *K. pneumoniae* isolates in our study, and this should be noted as evidence of widespread resistance pattern due to OXA-48 carbapenemase in the community of Turkey [11]. In Turkey, OXA-48 carbapenemase was first reported among *K. pneumoniae* in

2008, and since then *K. pneumoniae* with OXA-48 carbapenemase has become endemic [12].

We further analyzed the MLST typing profiles. Seven STs were detected by using the MLST database (Table 2). The most common clone in City 1 university hospital was found to be ST14 strain which constitutes 82% of the strains and was located in CC14, one of the international clones described previously [13]. There was no correlation between clones and the clinics in which they were isolated. The most common carbapenem resistance genes were bla_{NDM-1} and bla_{OXA-48}, whereas the colistin resistance gene could not be detected in the same hospital. The most common clone in City 2 university hospital was found to be ST2096 strain which constitutes 96% of the strains. There was no correlation between clones and the clinics in which they were isolated. We observed that some of the prevalent isolates with the same STs (ST14 for City 1 and ST2096 for City 2) were from patients who were hospitalized in the same period. bla_{OXA-48} were detected in all strains, whereas the other carbapenem and colistin resistance genes were not detected in City 2 university hospital (Table 2 and Figure 3).

Two index isolates found in this study, one from City 1 university hospital and the other from City 2 university hospital, ST14 and ST2096 respectively, are in the same clonal complex, CC14. Outbreaks with isolates belonging to the same clonal complex occurred in two different hospitals which were 600 km apart from each other. Recently, a total of 39 NDM-1 producing *Klebsiella pneumonia* isolates from India (n = 22), United Kingdom (n = 13), and Sweden (n = 4) were studied for their MLST types. The results of the study indicated that the most common ST type was ST14, and out of 39 isolates 13 were ST14 (33%) even though a diversity of ST clones in each country was observed. In addition, the authors stressed the importance of ST types especially ST14 for dissemination of $bla_{\text{NDM-1}}$ [14]. In other countries dissemination of carbapenem resistance with ST14 were reported. In USA ST14 isolates carrying both bla_{NDM} and $bla_{\text{OXA-48}}$ carbapenemase genes were reported [15]. In United Arab Emirates the most common bla_{NDM} carrying isolates was found to be ST14 and these isolates were from outbreaks in hospitals between 2009 and 2013. Interestingly, these NDM producing isolates also produced OXA48, and carbapenem resistance was mostly associated with colistin resistance (31.4%) [16]. Similarly, in our study presence of $bla_{\text{NDM-1}}$ was associated mostly with presence of $bla_{\text{OXA-48}}$. The most common ST among carbapenem resistant isolates in City 1 was ST14 and 86% (n = 12/14) of these isolates carried bla_{NDM}.

The clone ST2096 was the common clone in City 2 Medical Faculty Hospital. City 2 clone ST2096 was reported in a recent study from India [17]. Although few reports were published about ST2096, ability of this clone to make an outbreak in a hospital is confirmed with our study. In our study, ST14, ST79, and ST2096 were found to be in the same clonal complex, CC14. In the present study, we determined MLST types of carbapenem resistant *K. pneumoniae* isolates from two different centers. Although the most common ST types were different in these two hospitals, ST types were clustered in CC14. To the best of our knowledge, this is the first report of outbreaks caused by ST14 and ST-2096 in Turkey.

Source of Funds:
No external funding was received.

Declaration of Interest:
The authors have no declarations of interest to report.

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