JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Epidemic *Klebsiella pneumoniae* ST258 incidence in ICU patients admitted to a university hospital in Istanbul

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Abstract

Introduction: *Klebsiella pneumoniae* sequence type 258 (ST258) strains are globally distributed multi-drug resistant pathogens and can spread rapidly throughout the world, causing severe healthcare-associated invasive infections with limited antimicrobial treatment options. The aim of this study was to reveal the incidence of *Klebsiella pneumoniae* ST258 strains among the intensive care unit patients in a university hospital in Istanbul.

Methodology: Consecutive nonreplicated 83 *K. pneumoniae* strains were isolated from various clinical samples of intensive care unit patients admitted to a university hospital in Istanbul, between November 2016 to December 2018. Bacterial identifications were performed via VITEK2. Antimicrobial susceptibility tests were conducted with Kirby Bauer's disc diffusion test except for colistin which was performed with broth microdilution. Real-time PCR method was utilized in order to reveal ST258 positivity among the strains.

Results: Antimicrobial susceptibility results revealed that 56 (67%) *K. pneumoniae* strains were carbapenem-resistant. Real-time PCR results demonstrated that 15 out of 83 (18%) *K.pneumoniae* strain were ST258. According to antimicrobial susceptibility test results of ST258 strains, 8 were found as carbapenem-resistant whereas 7 were found as carbapenem susceptible. 3 out of 8 (37.5%) carbapenem-resistant ST258 strains were found as resistant against all antibiotics tested.

Conclusions: Our study revealed that *K. pneumoniae* ST258 which caused severe infections worldwide so far has also spread to Istanbul. We believe that rapid molecular methods for monitorization of these clones are useful. our results showed that ST258 is not linked to a multi-resistant strain and suggested that it does not contribute to multi-resistance formation alone.

Key words: *Klebsiella pneumoniae*; ST258; antimicrobial resistance; ICU.

J Infect Dev Ctries 2021; 15(5):665-671. doi:10.3855/jidc.13430

(Received 09 July 2020 – Accepted 04 December 2020)

Introduction

Klebsiella pneumoniae are Gram-negative, encapsulated, nonmotile bacteria that are colonized on human mucosal surfaces [1]. Due to their ability to invade and disseminate to other tissues, it causes several types of life-threatening infections in humans [2,3]. *K. pneumoniae* strains have been known as the most frequent cause of multidrug-resistant gram-negative bacterial infections [4]. Moreover, in the intensive care unit (ICU), in case of any infection with multidrug-resistant bacteria, broad-spectrum antibiotics are needed for treatment and these infections associated with worse outcomes compared to infections due to susceptible strain [5]. The majority of the *K. pneumoniae* strains express carbapenemases that break down most of the beta-lactams [6]. Genes that code for carbapenemases often spread worldwide through clonal expansion and *K. pneumoniae* sequence type 258 (ST258) strains are considered as a "high-risk international clone" [6,7].

K. pneumoniae ST258 clones are globally distributed multi-drug resistant pathogens and have capacity to spread rapidly throughout the world, causing severe healthcare-associated invasive infections with limited antimicrobial treatment options [8,9]. These strains may share genetic features that predispose them to pathogenicity or increased transmissibility. Carbapenem resistance is frequent among the strains, thus infections with ST258 strains are treated with regimens containing colistin. However, colistin-resistant strains have been reported from different parts of the world which is one of the major concerns of public health [10,11]. Although there are studies revealing the prevalence of ST258 clones in different parts of the world, there is only one case report the presence of ST258 clone in Turkey [12]. However, there is not any study investigating the prevalence of the clone in our country. In light of this information, we aimed to reveal the incidence and antimicrobial susceptibility of *Klebsiella pneumoniae* ST258 strains among the intensive care unit patients in a university hospital in Istanbul for the first time.

Methodology

Consecutive nonreplicated 83 *K. pneumoniae* strains were isolated from various clinical samples of intensive care unit patients admitted to a university hospital in Istanbul, between November 2016 to December 2018. Bacterial identifications were conducted with VITEK2 automated system, using VITEK 2 Identification cards, which provide rapid, accurate species-level identification of clinically relevant bacteria (BioMerieux, Marcy L'Etoile, France). Mucoid and lactose positive colony forming, gram negative bacilli are suspected as *K. pneumonie*, and further identification performed with automated system using VITEK2 Gram-negative bacilli Identification test card (VITEK2 GN ID card). Except for colistin, all other antimicrobial susceptibility tests were performed with Kirby Bauers disc diffusion tests on Mueller-Hinton agar (BioMerieux, Marcy L'Etoile, France). Briefly, a density of 0.5 McFarland bacterial suspension was inoculated in Mueller–Hinton broth and streaked on Mueller-Hinton agar plates, then antibiotic discs were placed on plates and incubated at 37 ºC overnight to detect antimicrobial susceptibility tests except colistin. Colistin susceptibility performed by the broth microdilution method and the results were interpreted according to the EUCAST clinical breakpoints [13,14]. The following antibiotic discs were used, amoxicillin-clavulanic acid (20/10 µg, Cat: CT0223B), piperacillin-tazobactam (30/10 µg, Cat: CT1628B), ampicillin (25 µg, Cat: CT0004B), cefotaxime (30 µg, Cat: CT0166B), cefepime (30 µg, Cat: CT0771B), ceftriaxone (30µg, Cat: CT0417B), ceftazidime (30 µg, Cat: CT0412B), fosfomycin (50 µg, Cat: CT0183B), amikacin (30 µg, Cat: CT0107B), gentamicin (10 µg, Cat: CT0024B), imipenem (10 µg, Cat: CT0455B), meropenem (10 µg, Cat: CT0774B), ciprofloxacin (5 µg, Cat: CT0425B), levofloxacin (5 µg, Cat: CT1587B), trimethoprim-sulfamethoxazole (1.25/23.75 µg, Cat: CT0052B) and tigecycline (15 µg, Cat: CT1841B) (Oxoid, Basingstoke, UK).

All *K. pneumoniae* strains was collected and stored at -80 ºC using specific Microbank cryovials containing 20% glycerol (Pro-Lab, Texas, USA). A cryovial bead was inoculated 10 mL Tryptone Soya Broth (TSB, Cat: CM1016B Oxoid Basingstoke, UK) and incubated at 37 ºC overnight. One hundred µL inoculum from TSB, streaked on eosin methylene blue (EMB) agar incubated at 37 ºC overnight to obtain a single colony for DNA extraction. Real-time PCR method was performed in order to reveal ST258 positivity among the strains. In order to extract bacterial DNA, in a single colony of each strain's fresh overnight culture on eosin methylene blue (EMB) agar was suspended in 50 µL of ultrapure water. The suspension was heated at 95 ºC for 10 minutes and centrifuged at 14.000 rpm for 10 minutes. Thirty microliters of the supernatant were used as a DNA template for real-time PCR [15]. All DNA was stored at -80 ºC until processing. Previously designed primers targeting the pilv-l region in the following sequences 5′-TTGGAGCTGATCCTTGCTCT and 5′-TCGATCCATGCTGATGATGT were used to detect ST258 clones among the strains [8]. Real-time PCR amplification and melting curve analysis were performed using a LightCycler 480 II system with software version 1.5 (Roche Diagnostics, Mannheim, Germany). The real-time PCR mixture was prepared using the LightCycler 480 SYBR Green I Mastermix kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Cycling conditions for the ST258 assays were: initial denaturation for 5min at 95 °C and 40 cycles of 5 seconds at 95 °C and 10 seconds at 58 °C [8]. The fluorescence signal was measured at the end of each annealing step. Following amplification, a melting curve was generated by heating the PCR product to 95 °C with a ramp rate of 0.05 °C/s. Statistical analysis was performed with chi-square test on SPSS vers. 20 software (IBM, USA).

Results

Antimicrobial susceptibility results revealed that 56 out of 83 (67%) *K. pneumoniae* strains were carbapenem-resistant. It has been observed that all strains were resistant to ampicillin (100%). Also, 67 out of 83 (81%) *K. pneumoniae* strains are resistant to the third generation cephalosporins, which are usually used to treat *Enterobacteriaceae* infections. In addition to this, resistance rates against trimethoprim-sulfamethoxazole, tigecycline and colistin, which are therapeutic options used instead of beta-lactam antibiotics, were found as 63.86%, 26.67% and 20.0%, respectively. Also, 3 out of 8 (37.5%) carbapenem-resistant ST258 strains were found as resistant against all antibiotics tested. According to susceptibility results, colistin was found to be the best therapeutic choice for

Table 1. Distribution of antimicrobial susceptibility result of all the strains. Resistant: n (%).

	CR-Kp (n = 56)	CS-Kp (n = 27)	Total (n = 83)
Colistin	13 (23.21)	2 (7.41)	15 (18.07)
Tigecycline	20 (35.71)	3 (11.11)	23 (27.71)
Ampicilin	56 (100)	27 (100)	83 (100)
Gentamicin	39 (69.64)	4 (14.81)	43 (51.81)
Amoxicillin-Clavulanic Acid	56 (100)	16 (59.26)	72 (86.75)
Piperacillin-Tazobactam	55 (98.21)	12 (44.44)	67 (80.72)
Ceftriaxone	55 (98.21)	14 (51.85)	69 (83.13)
Cefotaxime	55 (98.21)	14 (51.85)	69 (83.13)
Ceftazidime	54 (96.43)	10 (37.04)	64 (77.11)
Amikacin	41 (73.21)	2 (7.41)	43 (51.81)
Ciprofloxacin	49 (87.50)	8 (29.63)	57 (68.67)
Levofloxacin	49 (87.50)	8 (29.63)	57 (68.67)
Trimethoprim-Sulfamethoxazole	41 (73.21)	12 (44.44)	53 (63.86)
Imipenem	56 (100)	0 (0)	56 (67.47)
Meropenem	56 (100)	0 (0)	56 (67.47)
Cefepime	54 (96.43)	6 (22.22)	60 (72.29)

CRKp : Carbapenem resistant *K. pneumoniae*; CS-Kp: Carbapenem susceptible *K. Pneumoniae.*

all patients which was followed by tigecycline, amikacin and gentamicin. Antimicrobial susceptibility results of all strains were given in Table 1.

Real-time PCR results demonstrated that 15 out of 83 (18%) *K. pneumoniae* strain were ST258. According to antimicrobial susceptibility test results of ST258 strains, 8 were found as carbapenem-resistant whereas 7 were found as carbapenem susceptible. There was no statistically significant difference between ST258 incidence and carbapenem resistance ($p > 0.05$). Moreover, in three out of eight (37.5%) ST258 positive Carbapenem-resistant *K. pneumoniae* (CR-Kp) strains were colistin-resistant whereas there was not encountered any colistin-resistant strains among ST258 Carbapenem sensitive *K. pneumoniae* (CS-Kp). In other words, in three out of 15 (20%) ST258 strains carbapenem and colistin resistance co-existed. Table 2 and Figure 1 demonstrates the antimicrobial susceptibility results of ST258 positive stains.

In addition to this, when the data of patients infected with *Klebsiella pneumoniae* ST258 strains were

Figure 1. Antimicrobial resistance rate of the ST258 clones in CR-Kp and CS-Kp.

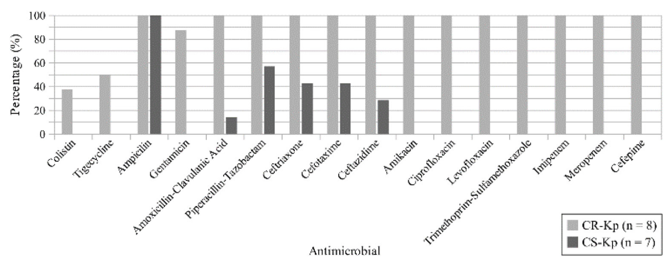

Table 2. Antimicrobial susceptibility result of the ST258 clone positive strains. Resistant: n(%).

ST258 positive	CR-Kp (n = 8)	CS-Kp (n = 7)	Total (n = 15)
Colistin	3 (37.50)	0 (0)	3 (20)
Tigecycline	4 (50)	0 (0)	4 (26.67)
Ampicilin	8 (100)	7 (100)	15 (100)
Gentamicin	7 (87.50)	0 (0)	7 (46.67)
Amoxicillin-Clavulanic Acid	8 (100)	1 (14.28)	9 (60)
Piperacillin-Tazobactam	8 (100)	4 (57.14)	12 (80)
Ceftriaxone	8 (100)	3 (42.85)	11 (73.34)
Cefotaxime	8 (100)	3 (42.85)	11 (73.34)
Ceftazidime	8 (100)	2 (28.57)	10 (66.67)
Amikacin	8 (100)	0 (0)	8 (53.34)
Ciprofloxacin	8 (100)	0 (0)	8 (53.34)
Levofloxacin	8 (100)	0 (0)	8 (53.34)
Trimethoprim-Sulfamethoxazole	8 (100)	0 (0)	8 (53.34)
Imipenem	8 (100)	0 (0)	8 (53.34)
Meropenem	8 (100)	0 (0)	8 (53.34)
Cefepime	8 (100)	0 (0)	8 (53.34)

CR-Kp : Carbapenem resistant *K. pneumoniae*; CS-Kp : Carbapenem susceptible *K. Pneumoniae.*

analyzed, it was noticed that the first treatment that some patients received contradicted with the in-vitro antimicrobial susceptibility results of the clone (Table 3). Six patients infected with ST258 received piperacillin and tazobactam combination therapy, however, in-vitro antimicrobial susceptibility tests revealed that only two strains were susceptible to the antibiotic in interest.

Discussion

Antimicrobial resistance is an important worldwide problem in the treatment of diseases caused by resistant bacteria. *Klebsiella pneumoniae* is defined as a member of the ESKAPE group microorganisms which comes from their ability to "escape" from the effects of antimicrobial drugs [16]. Therefore, *K. pneumoniae* is known to be an important cause of morbidity and mortality among hospital-acquired and long-term care-related infections [17].

Over the last few decades, there has been a concerning increase in the resistance of *K. pneumoniae* strains to a wide range of antibiotics. Expression of extended spectrum beta-lactamases (ESBLs) and carbapenemases are two major types of antimicrobial resistance mechanisms mainly observed in these strains [1,2]. The prevalence of multiple antibiotic-resistant strains such as CR-Kp has increased in recent years and CR-Kp has become a major public health problem worldwide, as carbapenems are the first-line therapy for infections caused by *K. pneumoniae*, especially ESBL producers [16,18]. Although not all the CR-Kp strains belonging to ST258 clone, previous studies revealed that there is a strong relationship between carbapenem resistance and ST258 clone. In addition to carbapenem

Table 3. Patients information isolated ST258 *Klebsiella pneumoniae* strains.

	Sample	Age	Gender	Phenotypically carbapenem susceptibility	Disease	Medication	Survive
P1	Sputum	31	M	CR	Rheumatic Valvular Heart Disease	Tygecycline	Remission or clinical improvement
P2	Catheter	45	M	CR	Lung Cancer, Pneumoniae	Levofloxacin	Remission or clinical improvement
P3	Blood	57	M	CR	Alcoholic Cirrhosis	Meropenem	Death*
P4	Blood	38	M	CR	Heart Failure	Colistin+Meropenem	Remission or clinical improvement
P5	Tracheal Aspirate	78	M	CS	Heart Failure	Piperacillin-Tazobactam	Death*
P6	CSF	57	M	CR	Coronary Heart Disease	Not Used	Death*
P7	Tissue	79	F	CR	Chronic Renal Failure + Ileus	Colistin	Remission or clinical improvement
P8	Urine	38	F	CR	Parathyroid Ca + Pulmonary Emboli	Piperacillin-Tazobactam	Remission or clinical improvement
P9	Blood	53	M	CS	Intracranial Hemorragy	Imipenem	Remission or clinical improvement
P10	Blood	79	F	CR	Chronic Renal Failure + Ileus	Colistin	Remission or clinical improvement
P11	Blood	94	F	CS	Chronic Lymphocytic Leukemia	Piperacillin-Tazobactam	Remission or clinical improvement
P12	Blood	41	M	CS	Intracranial Hemorragy	Piperacillin-Tazobactam	Death*
P13	Blood	76	M	CS	Prostat Ca	Ceftazidime	Death*
P14	Blood	94	F	CS	Chronic Lymphocytic Leukemia	Piperacillin-Tazobactam	Remission or clinical improvement
P15	Blood	34	F	CS	Colon Ca	Piperacillin-Tazobactam	Death*

*Death (not attributable to infection).

resistance, ST258 positive *K. pneumoniae* strains are known as resistant to all β-lactam antibiotics, also typically have plasmid-derived genes that encode aminoglycoside modifying enzymes and chromosomal mutations that confer fluoroquinolone-resistance, that make them multi-drug resistant strains [16-19]. Furthermore, treating CR-Kp infections is a major clinical challenge, due in part to limited antibiotic options. In spite of being a major health concern worldwide, the data related to the molecular epidemiology and molecular characteristics of these strains are insufficient in our country. When studies investigating the prevalence of ST258 clones among carbapenem-resistant *K. pneumoniae* (CR-Kp) strains were examined, Ocampo *et al.* reported 37.8% (73 out of 193 strains) ST258 positivity in their study conducted in Colombia in 2016 [19]. Also, Bonura *et al.* reported 40% (37 out of 94 strains) ST258 positivity among CR-Kp strains in Italy in 2015 [20]. In 2017, Satlin *et al.* reported that 77 out of 92 (84%) CR-Kp strains were ST258 [21]. In our study, for the first time in our country, we detected 8 (14.2%) strains belonging ST258 clone, among 56 CR-Kp strains.

Mavroidi *et al*. studied with *K. pneumoniae* strains isolated from intensive care units in Greece in 2016 and reported that 18 of 19 colistin-resistant CR-Kp strains were ST258 positive [22]. Moreover, Bogdanovich *et al*. reported that all five colistin-resistant CR-Kp strains were ST258 positive in their study conducted in 2011 [11]. Lomonaco *et al*. did not detect ST258 clone in any of the 10 multi-drug resistant *K. pneumoniae* strains and reported that these strains were belonging to different sequence types in the study conducted in Pakistan in 2018 [23]. In our study, we found colistin resistance rate as 37.5% (three out of eight) ST258 positive CR-Kp strains, which was not as high as it was found in Greece [22]. However, there was no statistically significant difference between ST258 incidence and carbapenem resistance, also ST258 was not linked to a multi-resistant strain.

In addition to this, there is only one case report on the ST258 clone, however, there is not any study revealing the prevalence of ST258 clone in our country [12]. Becker *et al.* reported ST258 positivity as 19.5% (66 out of 337), also reported ST258 positivity among CR-Kp strains as 28.9% (31 out of 107) in Germany in 2018. According to the results of their study, 35 out of 337 (10.3%) strains were carbapenem sensitive *K. pneumoniae* (CS-Kp) ST258 clone, whereas 31 out of 337 (9.1%) strains were CR-Kp ST258 clone [24].

Diago-Navarro *et al*. studied with 40 CR-Kp and 8 CS-Kp strains in 2014, detected ST258 clone in 80% (32 of 40 strains) of CR-Kp strains and 33% (3 of 8 strains) of CS-Kp strains [25]. Villa *et al*. reported that firstly they detected ST258 positive CR-Kp in intraabdominal abscess of a kidney transplant patient but during treatment with tigecycline they isolated ST258 positive CS-Kp in 2013. After examination of these two strains with the next generation sequence system, they reported that plasmid loss and carbapenem susceptibility could be seen after treatment with non-carbapenem antibiotics [26]. Diago-Navarro *et al*. reported 19% ST258 positivity among 300 *K. pneumoniae* strains in 2016 [27]. Moreover, they reported that 13% of ST258 positive strains were carbapenem susceptible due to the loss of carbapenemase gene which was attributed to ICU residence time and antibiotic use of ST258 positive patients, similarly to the study of Villa *et al*. [25-27]. The primers used in our study for detecting ST258 clones were targeting the pilv-1 region [8]. Adler *et al*. [8]. reported ST258 positivity in 9 CS-Kp strains when they used these primers in 2014 [8]. Similarly, the studies of Becker *et al*. [24], Diago-Navarro *et al*. [25], Diago-Navarro *et al*. [27] and Adler *et al*. [26], showed that ST258 clones were detected in carbapenem sensitive *K. pneumoniae* strains as well as CR-Kp. This may be a result of the loss of carbapenemase plasmids due to different antibiotic therapy regimens applied to the patients. However, although the previous studies reported the loss of carbapenemase plasmid, it is seen that majority of the studies on the ST258 clone have been tried to associate the clone only with carbapenem-resistant strains and CS-Kp strains were ignored [19-21]. In the study of Kontopidou *et al*. [28], conducted in Greece in 2014, it has been reported that they used beta-lactam inhibitors for the first 10 days as the first treatment option in ICU patients infected with CR-Kp which were strongly associated with bacteremia [28]. Clancy *et al*. [29]. reported that piperacillin-tazobactam was the first therapeutic option given to transplant recipients in the first 30 days after the detection of CR-Kp-induced bacteremia [29]. Moreover, in 2016, Ocampo *et al*. [19] reported that piperacillin-tazobactam was the treatment for patients with CR-Kp, which was similar to other studies in 2016 [19]. Similarly, in our study, CR-Kp and CS-Kp strains were mostly associated with bacteremias and it was found that beta-lactam/beta-lactamase inhibitor combinations such as piperacillin-tazobactam were frequently given during the treatment of patients. However, it was noticed that the treatment was started empirically, without obtaining in-vitro susceptibility results. Patient survival rates can be increased by rapid detection and control of infection source and infectious agent-specific combination therapy, whereas inappropriate empirical treatments result in poor clinical outcomes in patients [30].

Conclusions

This molecular epidemiological study is the first study conducted in Turkey in order to reveal the prevalence of ST258 clone, which was reported in only one case study in 2014 [12], among *K. pneumoniae* strains isolated from intensive care unit patients from a university hospital in Istanbul. In addition to the presence of ST258 clone in CR-Kp strains, ST258 clone positivity was also found in CS-Kp strains. Our results showed that ST258 is not linked to a multi-resistant strain and suggested that it does not contribute to multi-resistance formation alone. We believe that in order to be successful with empirical treatment in intensive care unit patients who are infected with MDR strains, the epidemiological data on *K. pneumoniae* strains with different clones that can rapidly disseminate multi-drug resistance should be developed with new multicenter and extensive studies.

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Conflict of interests: No conflict of interests is declared.