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Impact of the ST101 clone on fatality among patients with colistin-resistant *Klebsiella pneumoniae* infection

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Objectives: We describe the molecular characteristics of colistin resistance and its impact on patient mortality.

Methods: A prospective cohort study was performed in seven different Turkish hospitals. The genotype of each isolate was determined by MLST and repetitive extragenic palindromic PCR (rep-PCR). Alterations in *mgrB* were detected by sequencing. Upregulation of *pmrCAB*, *phoQ* and *pmrK* was quantified by RT–PCR. *mcr-1* and the genes encoding OXA-48, NDM-1 and KPC were amplified by PCR.

Results: A total of 115 patients diagnosed with colistin-resistant *K. pneumoniae* (ColR-Kp) infection were included. Patients were predominantly males (55%) with a median age of 63 (IQR 46–74) and the 30 day mortality rate was 61%. ST101 was the most common ST and accounted for 68 (59%) of the ColR-Kp. The 30 day mortality rate in patients with these isolates was 72%. In ST101, 94% (64/68) of the isolates had an altered *mgrB* gene, whereas the alteration occurred in 40% (19/47) of non-ST101 isolates. The OXA-48 and NDM-1 carbapenemases were found in 93 (81%) and 22 (19%) of the total 115 isolates, respectively. In multivariate analysis for the prediction of 30 day mortality, ST101 (OR 3.4, CI 1.46–8.15, $P = 0.005$) and ICU stay (OR 7.4, CI 2.23–29.61, $P = 0.002$) were found to be significantly associated covariates.

Conclusions: Besides ICU stay, ST101 was found to be a significant independent predictor of patient mortality among those infected with ColR-Kp. A significant association was detected between ST101 and OXA-48. ST101 may become a global threat in the dissemination of colistin resistance and the increased morbidity and mortality of *K. pneumoniae* infection.

Introduction

Emergence of MDR *Klebsiella pneumoniae* is a global concern. Carbapenemase-producing *K. pneumoniae* has rapidly spread across hospitals worldwide[1] and colistin has become the last-line treatment option for infections caused by this organism.[2] However, the emergence of colistin resistance is now limiting the success of treatment.[3] Colistin resistance rate in *K. pneumoniae* among healthcare-related bacteraemia in Turkey has increased from 6% in 2013 to 16% in 2015.[4]

The major mechanism of colistin resistance is modification of the cell wall lipopolysaccharide by addition of 4-amino-4-deoxyl-L-arabinose and/or phosphoethanolamine.[5] This modification can be initiated by chromosome-encoded or plasmid-transferred

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colistin resistance genes (*mcr-1* or *mcr-2*).[6–8] However, in *K. pneumoniae*, *mcr-1* positivity is very rare.[9] The resistance is primarily mediated by inactivation of the *mgrB* gene, which controls the PmrA/PmrB and PhoP/PhoQ two-component systems.[6,10,11] Although the molecular mechanisms of chromosome-mediated colistin resistance in *K. pneumoniae* have been reported in several studies, there are few reports showing differences in resistance mechanisms based on ST.[12]

K. pneumoniae ST101 belongs to clonal complex 11, which has been associated with different ESBL types, including CTX-M-15, OXA-48, NDM-1 and KPC.[13–16] It is now disseminated worldwide and carbapenem-resistant ST101 isolates have been identified from healthcare-associated infections (HAIs) in Europe, Asia, the USA and Latin America.[14,15,17,18] Currently, infections with colistin-resistant ST101 are emerging in Italy and Serbia.[12,19] In this study, we describe the molecular characteristics of colistin resistance and its impact on clinical outcomes, primarily patient mortality.

Methods

Study population and data collection

A prospective cohort study was performed between January 2015 and December 2016 in seven different hospitals in Ankara and Istanbul, Turkey. The participating centres were selected according to their research experience and multicentre collaboration capacity. Our study design met the criteria suggested by STROME-ID.[20] A study protocol was prepared for patients who had HAI with colistin-resistant *K. pneumoniae* (ColR-Kp). The HAIs were detected and recorded by the infection control team in each hospital. Among the HAIs, ColR-Kp were included and colistin-susceptible *K. pneumoniae* were excluded. In the protocol, patient demographic data, underlying diseases, type of infection, isolation site, blood biochemical parameters, predisposing factors such as having had an operation within last month, ICU admission, type of antimicrobial agents used for empirical and agent-specific therapy, duration of colistin therapy before isolation of colistin-resistant isolates, and carbapenem resistance were recorded on a standardized case form. The patients were followed for their outcome after isolation of colistin-resistant isolates. The 30 day mortality was defined as the mortality 30 days after isolation of the bacteria. Exclusion criteria were missing key data and subsequent episodes in the same patient. The Koc University Institutional Review Board approved the study (2015.048.IRB1.008).

Microbiological and molecular studies

K. pneumoniae was identified by using routine microbiological methods. As part of the routine surveillance of HAI, resistance to colistin among all *Klebsiella* isolates was tested by the Etest (bioMérieux, France) in routine microbiology laboratories and re-tested by the broth microdilution method in the central research laboratory. The isolates were considered resistant if the MIC was >2 mg/L using the broth dilution test.[21]

The *mgrB* gene was amplified using primers described previously[10] and sequenced with an automated ABI 3500 sequencer (Applied Biosystems, USA). The nucleotide sequences were manually trimmed to remove poor-quality reads and then mapped to a reference *mgrB* gene ±2 kb from the *K. pneumoniae* 1084 genome.[22] If alignment was partially successful, the un-aligned sequence (200–500 bp) was then aligned to a panel of transposons/transposases including ISs IS903B, IS1, IS5, IS903, ISEp1 and others. All work was done using CLC Main Workbench 7.6.1.

Expressions of *pmrA*, *pmrB*, *pmrC*, *phoQ* and *pmrK* were quantified by RT–PCR with *rpsl* being selected as housekeeping gene (LightCycler 480, Roche, Germany).[6,23] Colistin-susceptible *K. pneumoniae* clinical isolate CRK07 was used for normalization of the -ΔΔ*Ct* values.

Genotyping of the isolates was carried out 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). STs were determined using Applied Math Bionumerics V7.6 software. Repetitive extragenic palindromic PCR (rep-PCR) (Diversilab®) was used to determine the genetic relatedness of colistin-resistant isolates. Isolates that had a similarity index >95% were considered as clonally related. Genes for carbapenemases OXA-48, NDM-1 and KPC were searched for by multiplex PCR and amplicons were sequenced.[24] The *mcr-1* gene was screened for by PCR as described by Liu *et al.*[7]

Statistical analysis

Statistical analysis was performed using the statistical software package R. In univariate analyses, we used the Wilcoxon rank-sum test for continuous covariates and Fisher's exact test for discrete covariates. In multivariate analyses, logistic regression was performed using the variables that were detected to be significant in univariate analyses. All the results of statistical analysis are available as Supplementary data at *JAC* Online.

Results

A total of 115 patients with a ColR-Kp infection were included. During the study period, >249000 patients were hospitalized in the seven centres, 19800 patients were followed in ICUs and HAI was detected in 3397 patients. *K. pneumoniae* was isolated from 659 (19%) patients diagnosed with an HAI. One-hundred and twenty-six of these isolates were ColR-Kp and 11 isolates were excluded from the study according to our exclusion criteria. Among the study group of ColR-Kp, 30 day mortality was calculated to be 61%, whereas in colistin-susceptible *K. pneumoniae* the 30 day mortality rate was 23% (Figure 1). Patients were predominantly males (55%) and the median age of the patients was 63 (IQR 46–74). Bloodstream infections were diagnosed in 47 (41%) cases, ventilator-associated pneumonia (VAP) in 55 (48%) cases, surgical site and wound infections in 16 cases (14%) and urinary tract infections in 15 cases (13%). Sixty cases (52%) had an operation within 1 month of the infection and 98 cases (85%) stayed in the ICU.

All the ColR-Kp isolates were resistant to colistin, with MICs between 4 and 256 mg/L. Colistin was used for empirical therapy in 34 (30%) of the patients, and 38 (33%) patients received colistin in combination with meropenem or tigecycline for target-specific therapy, with a median duration of 12.5 days (between 1 day and 65 days).

In MLST analysis, ST101 was the most common ST and accounted for 68 (59%) of the ColR-Kp. The other commonly identified MLST STs were ST395 (13%), ST16 (5%) and ST47 (5%). ST could not be identified in three isolates. The ST101 proportion among STs was between 50% and 69% among the centres and the overall ST101 proportion was 59%. In rep-PCR, two clones that belonged to ST101 were observed. Thirty-four (30%) isolates from five hospitals were grouped in the first clone and 17 (15%) isolates from two hospitals were grouped in the second clone. The remaining isolates were non-clonally distributed (Supplementary data available at *JAC* Online).

The clades of the ColR-Kp strains isolated from both fatal and survived cases of the study were shown as a minimum spanning tree (Figure 2). The 30 day mortality rate was very diverse between the STs (Supplementary data available at *JAC* Online). The 30 day

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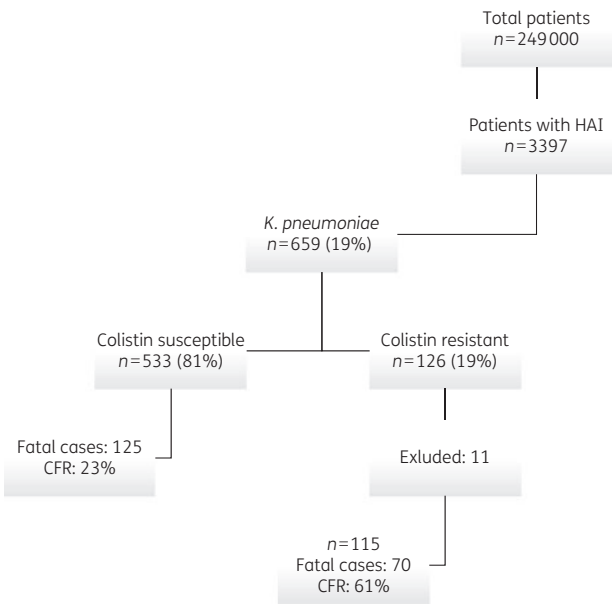


Figure 1. Flow chart for included ColR-Kp cases. CFR, case fatality rate.

mortality rate was 72% in ST101, while in other clades that had more than five cases ST395 and ST147 had a mortality of 73% and 50%, respectively.

Sequences of *mgrB* revealed that 83 of 115 isolates (72%) had a point mutation, deletions ($n = 6$) or an IS ($n = 77$) that interrupted the gene. The remaining 32 isolates (28%) had an intact *mgrB* gene. Three different IS types were found (IS5-like, IS1 and IS903), which were inserted at different positions in the gene. IS903 was the most common IS; it was detected in 36 (31%) of the isolates. In ST101, 94% (64/68) of the isolates had *mgrB* alteration with an IS or point mutation, whereas 40% (19/47) of the non-ST101 isolates had an altered *mgrB* gene ($P < 0.001$; Table 1). The ISs were found to be the most common cause of *mgrB* gene disruption in ST101 as compared with non-ST101 isolates (94% versus 74%, $P = 0.014$).

Upregulation of genes associated with colistin resistance, including *phoQ* (89%), *pmrK* (67%), *pmrA* (98%), *pmrB* (51%) and *pmrC* (86%), was seen in many isolates. The relative median increases were calculated to be 2.9-fold for *pmrC*, 5-fold for *pmrA*, 2-fold for *pmrB*, 2.5-fold for *phoQ* and 1.5-fold for *pmrK*. Overexpression of *phoQ*, *pmrK*, *pmrC*, *pmrA* and *pmrB* genes did not differ between ST101 and non-ST101 groups. No significant difference in expressions of these genes was found between the

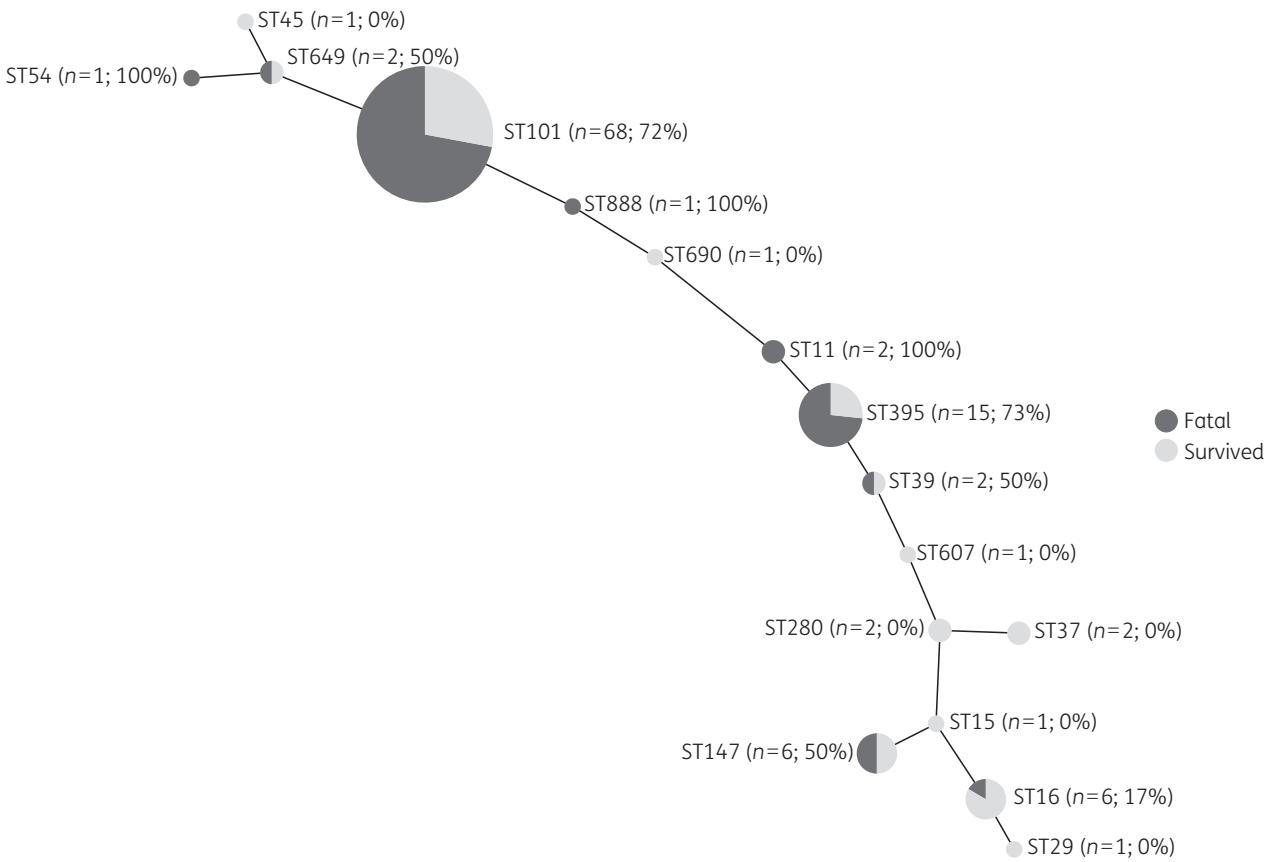


Figure 2. Minimum spanning tree of ColR-Kp by MLST ST and gene allele profiles. Each node within the tree represents a single ST. The area of the nodes is proportional to the number of isolates within each node; the proportions of fatal and survived cases are depicted as pie charts. The edge lengths between the node pairs are proportional to the number of different alleles out of seven MLST genes between two connected STs. Each node is labelled with the corresponding ST; the number of isolates and the 30 day mortality rate are given in parentheses.

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Table 1. Host and bacterial characteristics of patients infected with ST101 and non-ST101 ColR-Kp

	Total ($N = 115$)	ST101 ($N = 68$)	Non-ST101 ($N = 47$)	*P*
Host factors				
age, median, years (minimum–maximum)	63 (0–91)	63.5 (0–86)	60 (0–91)	0.548
female, *n* (%)	52 (45)	32 (47)	20 (43)	0.705
being in ICU, *n* (%)	98 (85)	58 (85)	40 (85)	1.00
operation history within 1 month, *n* (%)	60 (52)	34 (50)	26 (55)	0.704
VAP, *n* (%)	55 (48)	38 (56)	17 (36)	0.057
bacteraemia, *n* (%)	47 (41)	26 (38)	21 (45)	0.564
prior colistin use within the last 3 months, *n* (%)	53 (46)	28 (41)	25 (53)	0.342
duration of prior colistin use, days, median (minimum–maximum)	12.5 (1–65)	13 (1–65)	12 (2–60)	0.84
empirical carbapenem use, *n* (%)	54 (47)	29 (43)	25 (53)	0.342
30 day mortality, *n* (%)	70 (61)	49 (72)	21 (45)	0.004
more than two comorbidities, *n* (%)	34 (30)	19 (28)	15 (32)	0.681
Bacterial factors				
carbapenem resistance, *n* (%)	108 (94)	66 (97)	42 (89)	0.120
mgrB alteration, *n* (%)	83 (72)	64 (94)	19 (40)	<0.001
NDM-1, *n* (%)	22 (19)	0 (0)	22 (47)	<0.001
OXA-48, *n* (%)	93 (81)	65 (96)	28 (60)	<0.001

Table 2. Distribution of OXA-48 and NDM-1 among STs

	OXA-48 ($N = 93$)	NDM-1 ($N = 22$)
ST101 ($N = 68$), *n* (%)	65 (96)	0
ST395 ($N = 15$), *n* (%)	5 (33)	13 (87)
ST16 ($N = 6$), *n* (%)	4 (67)	0
ST147 ($N = 5$), *n* (%)	2 (40)	4 (80)
Other STs ($N = 21$), *n* (%)	17 (81)	5 (24)

isolates with an intact or disrupted *mgrB*. None of the isolates was found to be positive for *mcr-1*. We could not detect a significant association between upregulation of these genes and colistin exposure (Supplementary data available at *JAC* Online).

Carbapenem resistance rates were not significantly different between ST101 and non-ST101 STs ($P = 0.12$; Table 1). The OXA-48 and NDM-1 carbapenemase genes were found in 93 (81%) and 22 (19%) of all isolates, and 8 isolates were both NDM-1 and OXA-48 producers. Ninety-six percent of ST101 ($P < 0.001$) and 67% of ST16 isolates were OXA-48 carbapenemase producers, whereas 87% of ST395 and 80% of ST147 were NDM-1 carbapenemase producers (Table 2).

Among the clinical factors, the 30 day mortality rate was found to be significantly higher in ST101 than other STs ($P = 0.004$). In univariate analysis, VAP was found to be more common among the cases infected with ST101 than the patients with non-ST101 infections ($P = 0.057$). Colistin exposure and duration of colistin therapy before isolation of ColR-Kp did not differ between ST101 and non-ST101 groups ($P = 0.342$ and $P = 0.84$, respectively; Table 1). In multivariate analysis for the prediction of 30 day mortality, ST101 (OR 3.4, CI 1.46–8.15, $P = 0.005$) and being in the ICU (OR 7.4, CI 2.23–29.61, $P = 0.002$) were found to be significantly associated covariates (Table 3). Correlation plot analysis revealed that the most strongly associated covariates for 30 day mortality were being in the ICU (0.65), carbapenem resistance (0.6), OXA-48 (0.55) and ST101 (0.55) (Supplementary data available at *JAC* Online). In multivariate analysis, determinants of ST101 were detected to be OXA-48 (OR 6, CI 1.09–36.60, $P = 0.038$) and *mgrB* alteration (OR 12.9, CI 3.66–54.37, $P < 0.001$; Table 4).

Discussion

The worldwide increase in antibiotic resistance has necessitated the use of colistin as a last-resort antibiotic. Consequently colistin resistance has become a major health problem due to a lack of efficient alternative therapies.[25,26] In this large multicentre cohort study, we demonstrate the impact of ST101 on 30 day mortality among patients infected with ColR-Kp. ST101 *K. pneumoniae* was defined as a high-risk clone for the dissemination of carbapenem resistance in previous reports.[27] ST101 was found to be associated with OXA-48 and CTX-M-15 in Spain and Tunisia,[13,14] and with KPC-2 in Italy, the USA and Brazil.[15,28] Detection of ColR-Kp ST101 was reported from Italy and Serbia;[12,19,29] however, its impact on clinical outcomes was not described previously.

In multivariate analysis, infection with ST101 (OR 3.4, CI 1.46–8.15, $P = 0.005$) and being in an ICU (OR 7.4, CI 2.23–29.61, $P = 0.002$) were found to be independent predictors of 30 day mortality (Table 3). Being in an ICU as the predictor of mortality would not be surprising; however, it could indicate the severity of infection, which was associated with higher mortality. In a recent study, 13% of the carbapenem-resistant *K. pneumoniae* were reported to be resistant to colistin, and colistin resistance was reported to be associated with increased risk of in-hospital mortality.[26] Our study was done with ColR-Kp isolates, and ST101 seems to be associated with high virulence. In a recent study, ST101 was shown to have several virulence factors associated with capsule type, attachment and iron uptake.[30] In correlation plot analysis,

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Table 3. Predictors of 30 day mortality among patients infected with ColR-Kp

	Univariate analysis			Adjusted analysis[a]		
	OR	CI	*P*	OR	CI	*P*
Female	0.7	0.3–1.54	0.341	–	–	–
ICU stay	6.6	1.85–29.97	0.001	7.4	2.23–29.61	0.002
Bacteraemia	0.9	0.4–2.1	0.848	–	–	–
More than two comorbidities	0.5	0.22–1.29	0.145	–	–	–
VAP	2.3	0.99–5.33	0.038	1.6	0.71–3.86	0.249
Prior colistin use within the last 3 months	0.6	0.27–1.41	0.252	–	–	–
Carbapenem resistance	2.2	0.35–15.53	0.43	–	–	–
NDM-1	0.9	0.32–2.69	>0.999	–	–	–
OXA-48	1.4	0.48–3.9	0.628	–	–	–
ST101	3.2	1.36–7.52	0.004	3.4	1.46–8.15	0.005

[a]We included variables with a univariate *P* value of <0.1 for mortality in the multivariate analysis.

Table 4. Predictors of ST101 presence in patients infected with ColR-Kp

	Univariate analysis			Adjusted analysis[a]		
	OR	CI	*P*	OR	CI	*P*
Female	1.2	0.53–2.72	0.705	–	–	–
ICU stay	1	0.3–3.25	>0.999	–	–	–
Bacteraemia	0.8	0.34–1.75	0.564	–	–	–
Comorbidity	0.8	0.34–2.03	0.681	–	–	–
VAP	2.2	0.98–5.17	0.057	2.0	0.67–6.14	0.216
Prior colistin use within the last 3 months	0.6	0.27–1.39	0.254	–	–	–
Carbapenem resistance	3.9	0.6–42.49	0.12	–	–	–
Empirical carbapenem	0.7	0.29–1.48	0.342	–	–	–
NDM-1	0.0	0.00–0.08	<0.001	0.0	0.00–infinity	0.992
OXA-48	14.3	3.79–81.64	<0.001	6.0	1.09-36.60	0.038
mgrB alteration	22.8	6.82–100.59	<0.001	12.9	3.66–54.37	<0.001

[a]We included variables with a univariate *P* value of <0.1 for ST101 presence in the multivariate analysis.

infection with ColR-Kp ST101 (0.54) was found to be one of the strongest covariates associated with 30 day mortality (Supplementary data available at *JAC* Online). The *K. pneumoniae* ST101 clone was reported to have the highest number of virulence genes in the yersiniabactin siderophore cluster associated with iron uptake.[27] This cluster was previously shown to be associated with the ST258 clone.[31] The presence of yersiniabactin in *K. pneumoniae* is troublesome, because it is strongly linked to colonization in the respiratory tract.[31] In our study, ColR-Kp ST101 was found to be more common (56% versus 36%, $P = 0.057$) among the patients with pneumonia. In a study from Spain, the ST101/KPC-2 *K. pneumoniae* clone was detected in three out of the seven (42.9%) respiratory tract infections caused by *K. pneumoniae*.[27]

We detected a significant association between the presence of OXA-48 and ColR-Kp ST101. In previous studies, ST101 was reported as the predominant lineage responsible for the dissemination of carbapenemases by IncL plasmids carrying bla_{OXA-48}/$bla_{OXA-181}$ genes.[32] For colistin resistance both *mcr-1* and *mcr-2* genes[7] and *mcr*-independent plasmid-mediated resistance were also reported in a pair of colistin-susceptible *K. pneumoniae* and ColR-Kp from the same patient.[33] Moreover, an outbreak strain of *K. pneumoniae* ST101, which harbours a chromosomal copy of the ISEcp1-CTX-M-15 transposon was identified in Germany.[34] Thus, we postulate that alteration of *mgrB* in *K. pneumoniae* might be due to ISs transferred from OXA-48-carrying plasmids.

A study from China suggested that chromosomal mutations in *phoP/phoQ*, *pmrA/pmrB* and *mgrB* rather than *mcr-1* positivity might have an important role in increasing colistin resistance in *K. pneumoniae*.[9] In a study from Serbia, the ST101 clone was identified in 12 of 27 ColR-Kp isolates with an intact *mgrB* gene.[12] In our study, inactivation of *mgrB* by IS or point mutation was significantly higher in ST101 than non-ST101 isolates ($P = 0.014$). This finding implies that colistin resistance in ST101 may be irreversible, and ST101 could become a major resistant clone worldwide. Parallel to our work, recent studies have reported that colistin-resistant mutants created by *mgrB* insertional activation could be

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selected from other strains upon colistin exposure and this inactivation is stable even in the absence of colistin.[35] Interestingly, colistin use and its duration were not found to be associated with either ST101 or *mgrB* alteration in our study. Although the emergence of ColR-Kp *in vivo* is likely associated with prior colistin exposure in humans, there are reports of ColR-Kp in patients without any history of colistin therapy.[11,25] In *K. pneumoniae*, colistin resistance by inactivation of the *mgrB* gene did not show any significant biological cost,[35] in contrast to *Acinetobacter baumannii*.[36]

Besides ST101, another clone, ST395,[37] was found to be associated with high 30 day mortality; however, in our study the number of the cases infected with ST395 was much lower than the number infected with ST101. However, further studies are needed to understand this clone.

Upregulation of either the *pmrAB* or the *phoPQ* gene as a result of *mgrB* alteration was shown to contribute to colistin resistance in *K. pneumoniae*.[11,21] Although *mgrB* was more altered in ST101, we did not find higher expressions of these genes in ST101. The increased transcription of these genes in isolates with intact *mgrB* suggested to us that the expression of these genes might be under control of mechanisms other than *mgrB*.

This study has collected one of the largest number of clinical ColR-Kp isolates and characterized the clinical and molecular features. However, the sample size still could be one of the limitations that did not allow us to show the impact of ST clones other than ST101. In relation to this, we collected samples from two of the largest cities in Turkey, but studies with greater numbers of centres and a wider geographical distribution should be performed. We focused on the most frequently reported genes in the literature, and we did not explore unknown genes. Further molecular and clinical studies are needed on this topic to clarify the virulence, pathogenesis and therapy options. Another limitation of this study was related to using the Etest for detection of colistin resistance in the study centres. Thus, colistin-resistant isolates might have been misdiagnosed as susceptible. However, our molecular analysis was not affected because we included colistin-resistant isolates in our molecular studies and we re-tested MIC levels by broth microdilution. EUCAST released comments about the Etest at the end of the year 2016, but our study was started in 2015 and ended in 2016.[26]

Conclusions

In this study, we found that in addition to ICU stay, ST101 was a significant independent predictor of mortality among patients infected with ColR-Kp. A significant association was observed between ST101 and OXA-48. Overall, ST101 may be a global threat in the dissemination of colistin resistance and increased morbidity and mortality of *K. pneumoniae* infection.

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Transparency declarations

None to declare.

Supplementary data

Supplementary data are available at *JAC* Online.

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