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EPIDEMIOLOGY AND SURVEILLANCE

Molecular Epidemiology of Colistin-Resistant, Carbapenemase-Producing *Klebsiella pneumoniae* in Serbia from 2013 to 2016

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ABSTRACT Twenty-seven colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* isolates were identified from hospitals in Serbia. All isolates were $bla_{CTX-M-15}$ positive; ST101, ST888, ST437, ST336, and ST307 were bla_{OXA-48} positive; and ST340 was bla_{NDM-1} positive. ST307 had an insertion, and ST336 had a premature stop codon in the *mgrB* gene. Amino acid substitutions were detected in PmrAB of isolates ST101, ST888, ST336, and ST307. The *mcr-1* and *mcr-2* were not detected. An increase in *phoP*, *phoQ*, and *pmrK* gene transcription was detected for all sequence types.

KEYWORDS *Klebsiella pneumoniae*, carbapenem resistance, colistin resistance, molecular epidemiology

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Polymyxins are the treatment cornerstone for infections caused by carbapenem-resistant Gram-negative bacilli, including *Klebsiella pneumoniae*. Thus, the emergence of colistin-resistant strains among the multidrug-resistant *K. pneumoniae* is an inevitable result of the increased use of this antimicrobial agent. Outbreaks of colistin-resistant, carbapenemase-producing *K. pneumoniae* are especially worrisome and have been described in hospitals in many countries, such as Greece, South Korea, the United States, and France (1–5).

Twenty seven colistin- and carbapenem-resistant *K. pneumoniae* isolates recovered in three Serbian tertiary care hospitals and one private laboratory between 2013 and 2016, were analyzed in this study (Table 1). Isolates Kc3 and Kc4 originated from the same patient, as well as K3 and K9, but were isolated from different specimens or within a time span of 6 months, respectively. Twelve *K. pneumoniae* isolates came from a single hospital, The Clinical Center of Vojvodina, a university-affiliated medical center in Novi Sad, in the northern part of Serbia (October 2015 to February 2016). Four isolates were from The Clinical Center Niš, an academic medical center in Niš, in the southern part of the country, and 10 from a private laboratory in Belgrade, in central Serbia. The majority of the isolates were from adult patients, and there was no evidence of prior colistin administration for these patients (Table 1). The only pediatric isolate was isolate 11070, which was obtained from the large university-affiliated tertiary care pediatric hospital in Belgrade, the Mother and Child Health Care Institute of Serbia Dr. Vukan Čupić, from a 3-year-old patient from Ukraine. The child had previously received multiple courses of antibiotics to treat recurrent episodes of acute pyelonephritis and

TABLE 1 Characteristics of colistin-resistant, carbapenemase-producing *K. pneumoniae* isolates from Serbia[a]

Case	Medical setting	Isolate	Isolation date (day/mo/yr)	Clinical sample	Colistin MIC (μg/ml)	Imipenem/ meropenem MICs (μg/ml)	*mgrB* gene	PmrA/PmrB amino acid changes compared to colistin-susceptible strain	*bla* genes	PFGE genotype	MLST (ST)
1 (H)	CCN-N	Ni9	12/11/2013	Urine	>16	>8/>8	WT	–/–	$bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$	III	ST340
2 (H)	CCN-N	Ni21	09/01/2014	Urine	>16	>8/>8	WT	–/–	$bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$	III	ST340
3 (H)	CCN-N	Ni34	21/03/2014	Blood	>16	>8/>8	WT	–/–	$bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$	III	ST340
4 (H)	CCN-N	DM5	05/02/2014	Urine	>16	>8/>8	WT	–/–	$bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$	III	ST340
5 (H)	CCV-NS	Kc1	27/12/2015	Skin	16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
6 (H)	CCV-NS	Kc2	10/11/2015	Wound	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
7 (H)	CCV-NS	Kc3	27/12/2015	Wound	>16	>8/>8	WT	Ala217Val/Thr157Pro; Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
8 (H)	CCV-NS	Kc4	18/12/2015	Skin	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
9 (H)	CCV-NS	Kc5	06/01/2016	Skin	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
10 (H)	CCV-NS	Kc6	28/02/2016	Wound	16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
11 (H)	CCV-NS	Kc7	17/02/2016	Wound	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
12 (H)	CCV-NS	Kc8	20/11/2015	Wound	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
13 (H)	CCV-NS	Kc9	19/10/2015	BA	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
14 (H)	CCV-NS	Kc10	27/11/2015	Urine	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
15 (H)	CCV-NS	Kc11	10/08/2015	Skin	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
16 (H)	CCV-NS	Kc12	17/07/2015	Urine	16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	II	ST101
17 (O)	K-B	K1	28/10/2015	Urine	>16	8/8	WT	–/–	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	IV	ST437
18 (O)	K-B	K2	28/10/2015	Urine	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
19 (O)	K-B	K3	05/12/2015	Blood/CVC	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
20 (H)	K-B	K4	26/12/2015	Blood	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
21 (H)	K-B	K5	30/12/2015	Urine	>16	>8/>8	29 aa, truncated	Glu57Gly/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	V	ST336
22 (O)	K-B	K6	03/01/2016	Urine	16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
23 (O)	K-B	K7	2015	Urine	>16	8/8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
24 (O)	K-B	K8	2015	Urine	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
25 (O)	K-B	K9	01/02/2016	BA	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
26 (O)	K-B	K10	28/02/2016	Urine	>16	>8/>8	WT	Ala217Val/Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	VI	ST888
27 (H)	MCHCIS-B	11070	26/10/2015	TA	>16	>8/>8	IS*Kpn26*	Ala41Thr/Leu213Met; Gly256Arg	$bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$	I	ST307

[a]Abbreviations: H, hospitalized; O, outpatient; CCN-N, Clinical Center of Niš, Niš; CCV-NS, Clinical Center of Vojvodina, Novi Sad; K-B, Institute for Laboratory Diagnostics Konzilijum, Belgrade; MCHCIS-B, Mother and Child Health Care Institute of Serbia Dr. Vukan Čupić, Belgrade; BA, bronchial aspirate; CVC, central venous catheter; TA, tracheal aspirate.

had also been subjected to antibiotic prophylaxis, but there was no evidence of prior colistin administration.

Genetic relatedness among isolates was analyzed by pulsed-field gel electrophoresis (PFGE) of XbaI-restricted total genomic DNA according to a previously described protocol (6). PFGE testing revealed presence of six different genotypes. All isolates from northern Serbia (The Clinical Center of Vojvodina; Kc1-12) belonged to genotype II, and isolates from southern Serbia (The Clinical Center Niš; Ni9, Ni21, Ni34, and DM5) belonged to genotype III. Isolates from the private laboratory (Konzilijum, Belgrade) clustered in three different genotypes: genotype IV (K1), genotype V (K5), and genotype VI (K2, K3, K4, K6, K7, K8, K9, and K10). Pediatric isolate 11070 singled out and was designated genotype I. Multilocus sequence typing for representatives of each genotype was performed using primers and conditions described by Diancourt et al. (7). The determination of specific sequence types (STs) according to the obtained allelic profiles was accomplished using the database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) of the Institut Pasteur, Paris, France, and six different STs were identified (Table 1). Based on this analysis, the dominant ST was ST101 (genotype II), which encompassed 44.44% of the colistin-resistant isolates. These isolates were outbreak related and recovered from The Clinical Center of Vojvodina, Novi Sad, in the northern Serbia. This ST was followed by ST888 (genotype VI), which encompassed 29.63% of isolates. Isolates belonging to genotype III were designated ST340, those belonging to genotype IV were designated ST437, those belonging to genotype V were designated ST336, and those belonging to genotype I were designated ST307.

Antimicrobial susceptibility was determined by microdilution method according to the European Committee on Antimicrobial Susceptibility Testing recommendations (http://www.eucast.org) (8). The colistin MIC for all isolates was ≥16 μg/ml (Table 1). Antimicrobial susceptibility testing revealed that analyzed isolates were also resistant to carbapenems and that the MICs for imipenem and meropenem were ≥8 μg/ml. A PCR method was used to detect the carbapenemase-encoding genes bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48} (9–11), as well as $bla_{CTX-M-15}$ (12). $bla_{CTX-M-15}$ was detected in all isolates. Among the carbapenemase genes, the bla_{OXA-48} determinant was the most prevalent, being detected in 23 of 27 isolates (ST101, ST888, ST437, ST336, and ST307) (Table 1). bla_{OXA-48} has been commonly associated with ST101 worldwide and, according to the results of an 11-year (2001 to 2011) molecular epidemiologic study of bla_{OXA-48} in Europe and North Africa, ST101 is the most frequently observed sequence type (13). Among the carbapenem-resistant *K. pneumoniae* STs identified in this study, the emergence of colistin resistance had been already reported in KPC-2-producing ST101 (14) and bla_{KPC-2}- and bla_{CTX-M}-producing ST307 (15, 16). Carbapenem- and colistin-resistant isolates of ST437 have been reported previously (17). The bla_{NDM} gene was detected in ST340 (Table 1). Although ST340 strains carrying the bla_{NDM-1} gene had been described (18), colistin-resistant, NDM-1-producing isolates of this ST, to the best of our knowledge, have not yet been reported. The acquisition of colistin resistance by a NDM-1-producing *K. pneumoniae* strain highlights the risk of the emergence of panresistant strains. Colistin-resistant strains of OXA-48-producing ST888 and ST336 have not yet been found.

In order to reveal the molecular mechanism(s) of colistin resistance, the presence of *mcr-1* and *mcr-2* genes was analyzed in all colistin-resistant isolates from the collection by a previously described method (19, 20). Since the *mcr-1* and *mcr-2* genes were not found, we focused on other mechanisms of colistin resistance, specifically *mgrB* gene inactivation; the presence of the mutations in the *pmrA*, *pmrB*, *phoP*, *phoQ*, *crrA*, and *crrB* genes; and the upgraded expression of *phoP*, *phoQ*, and *pmrK* genes. The amplification of the *mgrB* gene was performed in all isolates by a previously described method (21). Sequence analysis of the *mgrB* gene showed that one isolate (ST307) generated amplicon that was larger than the one from *K. pneumoniae* IT977 (a control, colistin-susceptible isolate). Amplicon sequencing revealed that insertional inactivation had occurred in the coding region of the *K. pneumoniae* ST307 *mgrB* gene. Insertional inactivation occurred at nucleotide 75 and was raised by insertional sequence that

TABLE 2 Primers and conditions used in RT-qPCR[a]

Primer	Sequence (5′-3′)	Cycling conditions
phoP_F	ATTGAAGAGGTTGCCGCCCGC	95°C for 1 s, 52°C for 5 s, 72°C for 7 s
phoP_R	GCTTGATCGGCTGGTCATTCACC	95°C for 1 s, 52°C for 5 s, 72°C for 7 s
phoQ_F	ATATGCTGGCGAGATGGGAAAACGG	95°C for 1 s, 52°C for 5 s, 72°C for 7 s
phoQ_R	CCAGCCAGGGAACATCACGCT	95°C for 1 s, 52°C for 5 s, 72°C for 7 s
pmrK_FT	GCGGGCCATCAGGATCGACAGCG	95°C for 1 s, 65°C for 5 s, 72°C for 7 s
pmrK_RT	CGTTCTGGTACTACATCCCGTTCCTGA	95°C for 1 s, 65°C for 5 s, 72°C for 7 s
rpsL13_F	GCCGTACTTGGAGCGAGCCTG	95°C for 1 s, 52°C for 5 s, 72°C for 7 s
rpsL14_F	CCGTGGCGGTCGTGTTAAAGA	95°C for 1 s, 52°C for 5 s, 72°C for 7 s

[a]Reprinted from reference 21 with permission.

shared 99% of identity at the nucleotide level with IS*Kpn26* insertion sequence (IS*5* family of insertion sequences). The insertional sequence was identified using the ISfinder database (http://www-is.biotoul.fr) (22). Insertional inactivation was not detected in other STs from the study. However, ST336 had premature amber stop codon (TAG) due to a C-to-T change at position 88, which generates a truncated MgrB protein of 29 amino acids. Other STs had the wild-type *mgrB* gene, without any changes in nucleotide sequence that could result in change of protein synthesis or activity. Nucleotide sequences of genes and corresponding amino acid sequences of PmrA and PmrB proteins from all isolates were compared to those of the colistin-susceptible strain *K. pneumoniae* IT977, and the changes detected are shown in Table 1. The observed amino acid substitutions could have role in development of colistin resistance, but only Thr157Pro in the PmrB protein has been previously described (23). No amino acid substitutions were detected in PhoP or PhoQ protein. The *crrA* and *crrB* genes were found only in ST340, ST336 and ST307, but amino acid substitutions were not detected compared to *K. pneumoniae* available in GenBank.

Reverse transcription-quantitative PCR (RT-qPCR) was used to determine the expression levels of the *phoP*, *phoQ*, and *pmrK* genes. Expression of the *rpsL* gene represented an internal control. The primers and conditions used for the RT-qPCR analyses are listed in Table 2 (21). Normalization was done against the *rpsL* gene using the $\Delta\Delta C_T$ method (relative) (24), and the values obtained were then normalized against those detected in the colistin-susceptible isolate IT977. Analysis of *phoP* and *phoQ* transcription in ST307 with the inactivated *mgrB* gene revealed a 7.8-fold increase for the *phoP* gene and a 6.8-fold increase for the *phoQ* gene (Fig. 1). ST336, with a truncated MgrB, underwent 8- and 11-fold increases, respectively, in *phoP* and *phoQ* gene transcription. Although

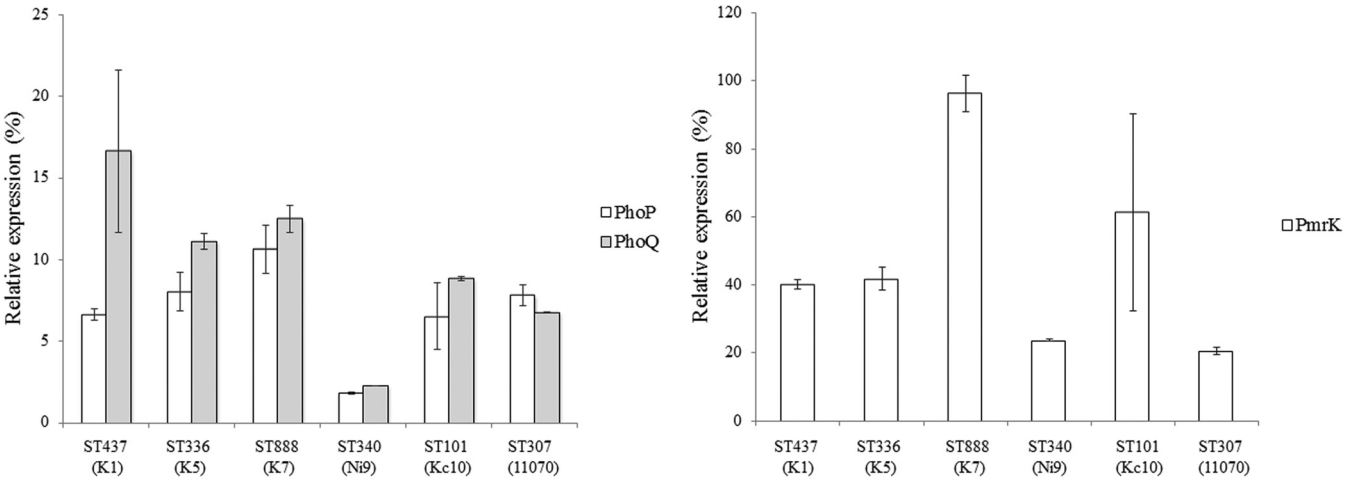

FIG 1 Relative expression of the *phoP*, *phoQ*, and *pmrK* genes in different colistin-resistant, carbapenemase-producing *K. pneumonia* STs isolated in Serbia. The values and standard deviations represent means from three independent experiments. The percent value represents the increase in gene expression relative to values observed for colistin-susceptible *K. pneumoniae* IT977.

other analyzed strains did not undergo insertional inactivation of the *mgrB* gene, the expression of the *phoP* and *phoQ* genes was elevated and ranged from an 1.8-fold increase in *phoP* gene expression in ST340 up to a 16.3-fold increase in *phoQ* gene expression in ST437 (Fig. 1). Moreover, analysis of transcription of the *pmrK* gene, which belongs to the *pmrHFIJKLM* operon, revealed a significant increase in transcription levels that varied from a 20.6-fold increase in ST307 to a 96.2-fold increase in ST888 (Fig. 1).

Although alterations in the *mgrB* gene nucleotide sequence are the most common cause of colistin resistance in *K. pneumoniae* (21, 25–28), we detected here the presence of such changes in only two isolates (ST336 and ST307), while the others had a wild-type nucleotide *mgrB* gene sequence. In addition, mutations leading to amino acid substitutions in PmrA and/or PmrB could have role in colistin resistance development in 22 of 27 isolates. However, the absence of changes in genes associated with colistin resistance for ST340 and ST437 could indicate that there are other regulators of PhoPQ regulatory system in *K. pneumoniae*, considering that such proteins have already been identified in *Escherichia coli*, *Shigella* sp., and *Salmonella enterica* serovar Typhimurium. Since these regulators are not conserved among *Enterobacteriaceae*, PhoPQ regulator(s) specific for *K. pneumoniae* may exist (29).

Accession numbers. The nucleotide sequence of the *mgrB* gene obtained from *K. pneumoniae* ST307 and ST336 are available in the European Nucleotide Archive under accession numbers LT635644 and LT635643, respectively. Other nucleotide sequences analyzed in this study are available from GenBank (KY586987 to KY587110).

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