COLISTIN RESISTANCE OF CARBAPENEM-RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS: MOLECULAR MECHANISMS AND BACTERIAL FITNESS

Shamina OV[1] ✉, Kryzhanovskaya OA[1], Lazareva AV[1], Alyabieva NM[1], Mayanskiy NA[2]

[1] National Medical Research Center for Children's Health, Moscow, Russia

[2] Pirogov Russian National Research Medical University, Moscow, Russia

The increasing use of colistin in the clinic has led to the emergence and spread of colistin resistance. According to the literature, antibiotic resistance can have a metabolic cost, resulting in poor adaptation and survival, i.e. reduced bacterial fitness. The aim of this study was to investigate molecular mechanisms underlying resistance to colistin and their effect on the bacterial fitness of carbapenem-resistant (carba-R) strains of *K. pneumoniae* isolated from the patients of Moscow hospitals in 2012–2017. Of 159 analyzed carba-R isolates, 71 (45%) were resistant to colistin (minimum inhibitory concentration over 2 mg/L). By conducting Sanger sequencing, we were able to identify the mechanisms underlying colistin resistance in 26 (37%) isolates. Growth curves were constructed by measuring optical density at 600 nm wavelength for 15 hours. The competitive growth of colistin-resistant (col-R) *K. pneumoniae* isolates was assessed relative to the colistin-susceptible (col-S) isolate. Col-R and col-S cultures harvested in the exponential phase were combined at the ratio of 1:1, incubated in the Luria-Bertani medium and plated onto Luria-Bertani agar plates with 10 mg/L colistin and without it. The competition index was calculated as the ratio of grown col-R and col-S colonies. Resistance to colistin did not affect the growth kinetics of *K. pneumoniae*, but did reduce the competitive ability of the bacteria as compared to the col-S isolates. However, some col-R isolates were more competitive than the col-S strains of the same sequence type. Further research is needed to elucidate the effects of colistin resistance on bacterial fitness.

Keywords: *Klebsiella pneumoniae*, bacterial fitness, colistin resistance, *mgrB*, sequence type

Funding: the study was supported by the Russian Science Foundation (Project ID 20-15-00235).

Acknowledgements: the authors thank Polikarpova SV of Filatov City Clinical Hospital № 15 and Karaseva OV of the Research Institute of Emergency Pediatric Surgery and Traumatology for *K. pneumoniae* isolates.

Author contribution: Shamina OV planned and conducted the study, analyzed the literature, analyzed and interpreted the obtained data, and wrote the manuscript; Kryzhanovskaya OA, Lazareva AV, Alyabieva NM planned and conducted the study; Mayanskiy NA planned, conducted and supervised the study, analyzed the literature, collected, analyzed and interpreted the obtained data, and wrote the manuscript.

Compliance with ethical standards: the study was carried out following the safety guidelines on the manipulations with pathogens of hazard groups III and IV.

✉ **Correspondence should be addressed:** Olga V. Shamina
Lomonosovskiy prospect, 2, str. 1, Moscow, 119991; olga.shamina@inbox.ru

Received: 18.05.2020 **Accepted:** 02.06.2020 **Published online:** 12.06.2020

DOI: 10.24075/brsmu.2020.032

УСТОЙЧИВОСТЬ КАРБАПЕНЕМРЕЗИСТЕНТНЫХ ШТАММОВ *KLEBSIELLA PNEUMONIAE* К КОЛИСТИНУ: МОЛЕКУЛЯРНЫЕ МЕХАНИЗМЫ И БАКТЕРИАЛЬНЫЙ ФИТНЕС

О. В. Шамина[1] ✉, О. А. Крыжановская[1], А. В. Лазарева[1], Н. М. Алябьева[1], Н. А. Маянский[2]

[1] Национальный медицинский исследовательский центр здоровья детей, Москва, Россия

[2] Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва, Россия

В последние годы широкое использование колистина в лечении инфекционных заболеваний привело к появлению и распространению колистинрезистентности. По данным литературы, формирование устойчивости может приводить к затратам внутренних биологических ресурсов и снижению уровня приспособленности и поддержания жизнедеятельности (бактериального фитнеса). Целью исследования было изучить молекулярные механизмы резистентности к колистину и их влияние на бактериальный фитнес карбапенемрезистентных (карба-Р) штаммов *K. pneumoniae*, выделенных у пациентов в г. Москве в 2012–2017 гг. Из 159 карба-Р-изолятов 71 изолят (45%) обладал резистентностью к колистину (минимальная подавляющая концентрация больше 2 мг/л); секвенирование по методу Сенгера позволило обнаружить механизмы устойчивости у 26 (37%) изолятов. Кривые роста были построены путем измерения оптической плотности при длине волны 600 нм в течение 15 ч. Конкурентный рост колистинрезистентных (кол-Р) изолятов *K. pneumoniae* оценивали относительно колистинчувствительного (кол-Ч) изолята. Кол-Р- и кол-Ч-изоляты в экспоненциальной фазе роста смешивали в пропорции 1 : 1, инкубировали в среде Луриа–Бертани и затем наносили на агар Луриа–Бертани, содержащий 10 мг/л колистина, и без него. Индекс конкуренции рассчитывали как отношение выросших кол-Р- и кол-Ч-колоний. Резистентность к колистину не влияла на кинетику роста *K. pneumoniae*, но снижала конкурентоспособность относительно кол-Ч-изолята. Тем не менее были обнаружены кол-Р-изоляты с высоким уровнем конкурентоспособности по сравнению с кол-Ч-изолятами такого же сиквенс-типа. Таким образом, необходимы дальнейшие исследования влияния резистентности к колистину на бактериальный фитнес.

Ключевые слова: *Klebsiella pneumoniae*, бактериальный фитнес, колистинрезистентность, *mgrB*, сиквенс-тип

Финансирование: исследование выполнено при поддержке гранта Российского научного фонда (проект № 20-15-00235).

Благодарности: авторы благодарят С. В. Поликарпову из Городской клинической больницы № 15 имени О. М. Филатова и О. В. Карасеву из Научно-исследовательского института неотложной детской хирургии и травматологии за предоставление изолятов *K. pneumoniae*.

Вклад авторов: О. В. Шамина — планирование и проведение исследования, анализ литературы, сбор, анализ и интерпретация данных, подготовка текста публикации; О. А. Крыжановская, А. В. Лазарева, Н. М. Алябьева — планирование и проведение исследования; Н. А. Маянский — научное руководство, планирование и проведение исследования, анализ литературы, сбор, анализ и интерпретация данных, подготовка и редактирование рукописи.

Соблюдение этических стандартов: исследование было проведено с соблюдением всех правил безопасности работы с микроорганизмами III–IV групп патогенности.

✉ **Для корреспонденции:** Ольга Вячеславовна Шамина
Ломоносовский проспект, д. 2, стр. 1, г. Москва, 119991; olga.shamina@inbox.ru

Статья получена: 18.05.2020 **Статья принята к печати:** 02.06.2020 **Опубликована онлайн:** 12.06.2020

DOI: 10.24075/vrgmu.2020.032

Klebsiella pneumoniae is a common cause of infections that require medical attention [1]. The emergence and global spread of high-risk multidrug-resistant (MDR) *K. pneumoniae* sequence types is a worrying trend [2, 3]. Carbapenem-resistant (carba-R) *K. pneumoniae* are an especially serious concern because resistance to carbapenems often co-occurs with resistance to other antimicrobial drugs, which dramatically narrows the range of therapeutic options for *K. pneumoniae* infection. As revealed by multilocus sequence typing (MLST), the majority of carba-R isolates are represented by a small group of sequence types that universally dominate nosocomial populations [4, 5]. At present, the following sequence types are classed as globally disseminated: ST14/15, ST17/20, ST43, ST147, ST258, ST395 [5, 6], and ST307, which only recently has been recognized as clinically relevant [7].

The polycationic antibiotic colistin, also known as polymyxin E, retains activity against carba-R gram-negative microorganisms. However, its wide use in the clinic in the backdrop of rampant resistance to carbapenems has driven the emergence of colistin resistance [4, 8], which can significantly reduce the efficacy of antimicrobial therapy and result in increased mortality in patients infected with colistin-resistant (col-R) *K. pneumoniae* [9].

Resistance to colistin arises from the structural modification of bacterial lipopolysaccharides (LPS) that prevents the antibiotic from binding to the bacterial cell wall [10]. This modification is associated with alterations in the two-component PhoPQ/PmrAB system and its regulator MgrB caused by mutations in the *mgrB* gene, as well as with plasmid-borne *mcr* genes [8, 10].

Naturally, being able to thrive in the presence of an antibiotic, resistant strains have an advantage over susceptible strains; however, there is a biological cost to pay: resistant strains grow at a slower rate and are less competitive in the absence of selective pressure exerted by antibiotics, i.e. have lower bacterial fitness [11, 12] than their susceptible counterparts [13, 14]. Considering that resistance to colistin is linked to LPS modifications, which is the key component of the bacterial cell wall, colistin resistance might be associated with reduced bacterial fitness.

The aim of this study was to characterize the genotype of carba-R *K. pneumoniae* isolated from the inpatients of Surgery and Intensive Care Units of Moscow hospitals, describe molecular mechanisms underlying resistance to colistin and investigate the effect of colistin resistance on the growth kinetics and the competitive ability of this bacterial population.

METHODS

We analyzed 159 carba-R *K. pneumoniae* isolates (the minimum inhibitory concentrations (MIC) of meropenem and imipenem were > 8 mg/L and > 4 mg/L, respectively, as defined by EUCAST criteria) [15] with and without resistance to colistin that had been collected from the patients of Surgery and Intensive Care Units of Moscow hospitals in 2012 through 2017. Only one *K. pneumoniae* isolate per patient was included in the collection. Biological samples had been taken from normally sterile sites (blood, urine, cerebrospinal fluid), the respiratory tract (aspirates, sputum), the oropharyngeal cavity, stomas, wounds, and the anus.

MIC of meropenem, imipenem and tigecycline were determined by performing Etests (BioMerieux; France) on Mueller-Hinton agar plates (Bio Rad; France). Susceptibility to aminoglycosides (gentamicin, netilmicin, amikacin), ciprofloxacin, fosfomycin, cefotaxime, cefepime, and ceftazidime was evaluated using an automated VITEK 2 Compact instrument for bacterial identification and susceptibility testing (BioMerieux; France). Colistin MIC were measured by broth microdilution as recommended in the National Standards of the Russian Federation (GOST R ISO 20776-1-2010); colistin used in the experiments was a powder formulation. The ATCC 25922 strain of *Escherichia coli* served as a control. According to EUCAST, colistin susceptibility and resistance breakpoints for *K. pneumoniae* are ≤ 2 mg/L and > 2 mg/L, respectively [15].

Detection and/or Sanger sequencing of the *mcr-1*, *mgrB*, *pmrA*, *pmrB*, *phoP*, and *phoQ* genes and the analysis of amino acid sequences of the PmrA, PmrB, PhoP, and PhoQ proteins were carried out following the previously described protocols [16]. An *mcr-1*-positive strain of *E. coli* provided by the Research Institute of Antimicrobial Chemotherapy (Smolensk State Medical University, Russia) was used as a control for *mcr-1* detection. *K. pneumoniae* strains were genotyped by means of multilocus sequence typing (MLST) [17]. Insertions were identified using the ISfinder database [18].

Bacterial fitness was studied in a subset of colistin-susceptible (col-S) and colistin-resistant (col-R) *K. pneumoniae* isolates with disrupted and wild-type *mgrB*. The cultures were grown on Luria–Bertani agar (HiMedia Laboratories Pvt. Limited; India) for 24 h. Protocols for assessing bacterial fitness were previously described in [14]. One bacterial colony was suspended in LB and incubated in an orbital shaker incubator ES-20 (BioSan; Latvia) at 37 °C for 3 h at constant stirring at 250 rpm. Bacterial concentrations were measured using a Novocyte flow cytometer (ACEA Biosciences; USA).

To construct and compare growth curves for col-R and col-S strains, the obtained suspension was diluted to a concentration of 5×10^6 bacterial cells per 1 ml. The resulting suspension (250 µl) was plated on flat-bottom 96-well plates containing 0, 1, 4, 16, or 64 mg/L colistin and incubated in an Infinite 200 microplate reader (Tecan; Austria) at 37 °C for 15 h. Incubation was performed in 3 replicates for each strain. Every 30 min, the optical density of the incubated samples was measured at 600 nm (OD_{600}). Measurements were recorded in Magellan 6.6 software (Tecan; Austria). The area under the growth curve (AUGC) was an indicator of bacterial growth; it was calculated for the period between the beginning of exponential growth and the point when plateau was reached (Fig. 1). AUGC was expressed as OD_{600} per hour.

To evaluate the competitive ability of col-R and col-S *K. pneumoniae* isolates, the competition index (CI) was calculated. Briefly, the suspensions of col-R and col-S isolates were

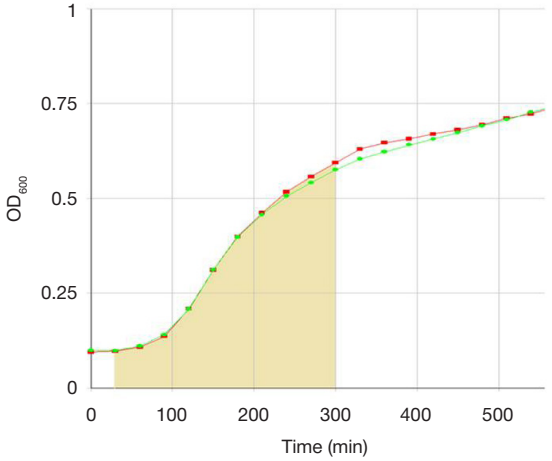

Fig. 1. Typical growth curves for *K. pneumoniae* isolates in the colistin-free culture medium. The shaded region on the graph represents an area under the growth curve (AUGC). The growth curve for col-S isolates is shown in red; the growth curve for col-R isolates is shown in green

Table 1. Genotypes, phenotypes and mechanisms underlying colistin resistance in carba-R *K. pneumoniae* isolates (n = 26)

Isolate ID	ST	Colistin MIC, mg/L	*mgrB* status [a]
69–77	23	128	IS*1A*, family IS-1 (+127/+128)
56–1790	307	64	IS*1R*, family IS–1 (+36/+37)
68–66–1	48	16	IS*Kpn14*, family IS–1 (+141/+142)
58–2876	48	128	IS*Kpn14*, family IS–1 (+141/+142)
58–3431	48	128	IS*Kpn14*, family IS–1 (+141/+142)
58–2966	48	512	IS*Kpn14*, family IS–1 (+141/+142)
56–1678	48	≥ 1024	IS*Kpn14*, family IS–1 (+141/+142)
56–1053	48	≥ 1024	IS*Kpn14*, family IS–1 (+141/+142)
71–1375	307	512	IS*Kpn14*, family IS–1 (+141/+142)
76–2089	377	512	IS*Kpn14*, family IS–1 (+141/+142)
64–574	307	256	IS*Kpn26*, family IS–5 (+74/+75)
4469	395	128	IS*Kpn26*, family IS–5 (+74/+75)
52–1659	395	256	IS*Kpn26*, family IS–5 (+74/+75)
58–1363	307	16	MITE*Kpn1*, family IS–5 (+74/+75)
55–148	307	64	MITE*Kpn1*, family IS–5 (+74/+75)
56–566	307	128	MITE*Kpn1*, family IS–5 (+74/+75)
58–1286	307	128	MITE*Kpn1*, family IS–5 (+74/+75)
56–613	307	512	MITE*Kpn1*, family IS–5 (+74/+75)
48–1594	307	≥ 1024	MITE*Kpn1*, family IS–5 (+74/+75)
78–296	37	16	Δ *mgrB* locus
37262	147	64	Δ *mgrB* locus
29423	70	128	Δ *mgrB* locus
36–2246	395	128	Δ *mgrB* locus
46–1574	307	128	Wild type [b]
48–2246	395	≥ 1024	Wild type [c]
56–410	48	128	Wild type [d]

Note: ST — sequence type; MIC — minimum inhibitory concentration; [a] — the position of the insertion sequence is specified in brackets; [b] — PmrB alteration (T157P); [c] — PmrA (A141T) and PmrB (L213M, G256R) alterations; [d] — PmrB alteration (deletion at 27–30 (QLIS)).

adjusted to 1.5 × 10^3 cells per 1 ml and combined at a 1 : 1 ratio (1.5 × 10^3 CFU per 1 ml for each strain). The mixture of col-R and col-S isolates and suspensions of unmixed col-S and col-R strains were grown in LB at 37 °C at 180 rpm for 16–18 h. Upon incubation, the suspensions were diluted 10^5-fold and plated on Petri dishes containing LB agar supplemented with 10 mg/L colistin or LB agar without colistin; plating was performed using an easySpiral automated spiral plater (Interscience; France). The cells were incubated at 37 °C for 16–18 h. CFU were counted using an automated Scan 500 colony counter (Interscience; France). CI was calculated as a ratio of col-R CFU in the LB dish with colistin to col-S CFU in the dish without colistin. CI < 1 was interpreted as reduced competitive ability of the resistant isolate, as compared to the susceptible isolate. The experiments were conducted in 3 replicates.

Statistical analysis was carried out in IBM SPSS Statistics 20.0 (IBM SPSS Inc; USA). Below, AUGC values and the number of colonies are presented as a median (P_{25}; P_{75}), CI is presented as a mean and a standard deviation. Differences in CI were evaluated using the Kruskal–Wallis test; pairwise comparisons were done using the Mann–Whitney U test. The differences were considered significant at $p < 0.05$.

RESULTS

Characterization of carba-R isolates of *K. pneumoniae*

All 159 carba-R *K. pneumoniae* isolates had an MDR-phenotype, i.e. were resistant to at least 3 classes of antimicrobial drugs. All studied strains were resistant to the third and fourth-generation cephalosporins and were highly resistant to ciprofloxacin (93%), fosfomycin (90%), netilmicin (82%), gentamicin (84%), amikacin (50%), and colistin (45%). The majority of carba-R *K. pneumoniae* isolates were susceptible to tigecycline; only 7% were resistant to this drug.

As revealed by MLST, the studied carba-R isolates were represented by 18 sequence types, of which only 5 dominated the collection, occurring in 86% of cases. Those included ST307 (n = 46, 29%), ST395 (n = 40, 25%), ST377 (n = 17, 10%), ST48 (n = 17, 10%), and ST23 (n = 16, 10%).

Mechanisms of colistin resistance

Resistance to colistin was observed in 71 (45%) carba-R *K. pneumoniae* isolates; for those isolates, colistin MIC varied from 4 to 1024 mg/L or was even higher. Investigation of

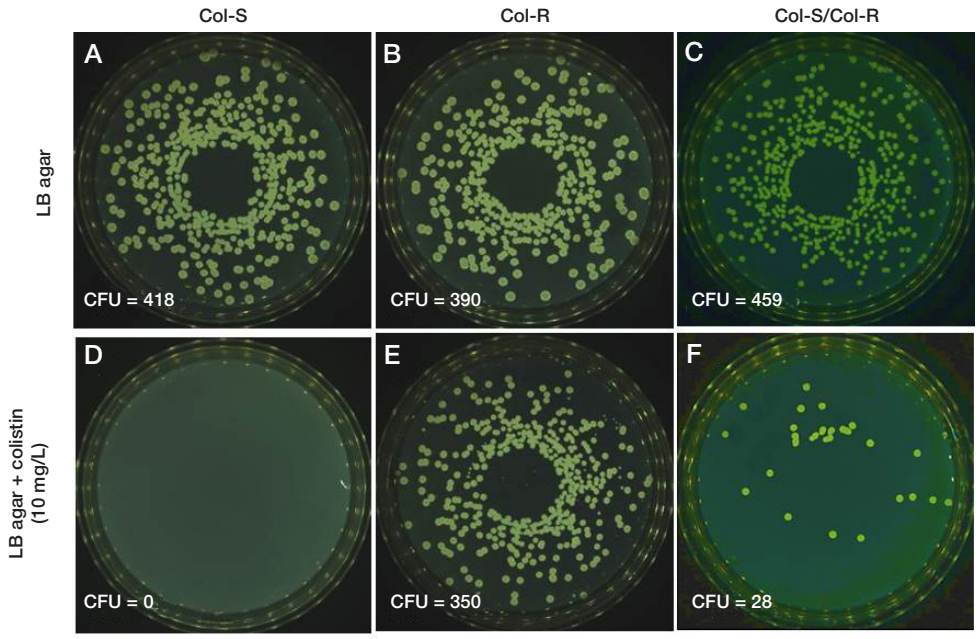

Fig. 2. Evaluation of the competitive ability of *K. pneumoniae* (a representative experiment). Col-S — colistin-susceptible isolates; col-R — colistin-resistant isolates; col-S /col-R — a mixture of susceptible and resistant strains; CFU — colony forming units. The photos of Petri dishes demonstrate the growth pattern for the col-S isolates (**A, D**), col-R isolates (**B, E**) and the mixture of col-R/col-S isolates (**C, F**) of *K. pneumoniae* on LB agar plates without colistin (**A–C**) and supplemented with 10 mg/L colistin (**D–F**). Numbers indicate the CFU count on each plate. The competition index (CI) is calculated as (the number of CFU on LB + colistin) divided by (the number of CFU on LB minus the number of CFU on LB + colistin), i.e. CFU F : (CFU C-CFU F)

molecular mechanisms underlying resistance to colistin was started with a search for the plasmid-borne gene *mcr-1*, which, according to the literature, is the most common cause of resistance [19]. We found that none of 71 col-R *K. pneumoniae* isolates carried the *mcr-1* gene.

Then, we went on to analyze the sequence integrity of the *mgrB* gene whose disruption might be associated with colistin resistance. Mutations in the *mgrB* gene were observed in 23 (32%) col-R isolates (Table 1). Deletion of the entire *mgrB* locus was detected in 4 (17%) isolates. In 13 (56%) isolates, there were insertions of 4 different types (IS1A, IS1R, IS*Kpn14*, and IS*Kpn26*), which occurred at different positions and represented the IS-1 and IS-5 families (Table 1). In 6 (26%) col-R isolates, the *mgrB* gene harbored a new mobile element (MITE*Kpn1*) described in our previous publication [16].

Summing up, the *mgrB* gene was wild-type in only 48 of 71 (68%) col-R *K. pneumoniae* isolates. Therefore, we had to continue looking for other mechanisms underlying colistin resistance. We analyzed the amino acid sequences of the proteins PmrA, PmrB, PhoP, and PhoQ in all 48 isolates. These proteins participate in LPS modification. Alterations in their sequences can cause resistance to colistin [10]. Significant alterations in PmrA and/or PmrB sequences were detected in 3 isolates from 3 different sequence types (ST307, ST395, ST48), with colistin MIC ranging from 128 to 1024 mg/L or being even higher (Table 1).

Effects of colistin resistance on bacterial fitness

In the absence of colistin, the growth kinetics of col-R and col-S *K. pneumoniae* did not differ significantly. Median AUGC values were 4.2 (3.9; 4.3) and 4.05 (3.9; 4.6) OD600 per 1 h, respectively (p = 0.842; Table 2). Addition of 1 mg/L colistin to the culture of col-S isolates caused AUGC to drop abruptly to 1.9 (0.95; 4.13) OD_{600} per 1 h (p = 0.065), whereas higher concentrations of colistin completely inhibited the growth of

Table 2. Effects of colistin resistance on the bacterial fitness (growth kinetics and competition index) of carba-R *K. pneumoniae* isolates

Isolates	Colistin MIC, mg/L; Me (P_{25}; P_{75})	AUC (OD_{600} per 1 H), Me (P_{25}; P_{75})					CI, mean (SD)
		Colistin concentration, mg/L					
		0	1	4	16	64	
Col-S (n = 6)	< 1 (< 1; < 1)	4.05 (3.9; 4.6)	1.9 (0.95; 4.13)	0 (0; 4.03)	0 (0; 0)	0 (0; 0)	n/a
Col-R (n = 32)	256 (128; 512)	4.2 (3.9; 4.3)[a]	4.1 (3.7; 4.2)[b]	3.9 (3.2; 4.15)[c]	3.3 (2.2; 3.45)[d]	0.9 (0; 3)[d]	0.15 (0.21)[f]
Of them:							
mgrB, disrupted (n = 15)	256 (128; 512)	4.1 (3.9; 4.2)	4 (3.9; 4.2)	3.9 (3.1; 4.1)	3.4 (0.9; 3.7)	1.1 (0; 3.3)	0.1 (0.1)[g]
mgrB, wild type (n = 17)	256 (96; 512)	4.3 (3.85; 4.4)[e]	4.2 (3.53; 4.25)[e]	3.8 (3.18; 4.25)[e]	3.25 (2.75; 3.43)[e]	0.9 (0; 3)[e]	0.19 (0.26)[h, i]

Note: MIC — minimum inhibitory concentration; AUGC — area under growth curve; Me — median; P_{25} and P_{75} — the 25th and 75th percentiles; CI — competition index; SD — standard deviation; col-S — colistin-susceptible isolates; col-R — colistin-resistant isolates; n/a — not applicable; [a] — p = 0.842 for comparison with col-S AUGC; [b] — p = 0.19 for comparison with col-R AUGC at 0 mg/L colistin; [c] — p = 0.016 for comparison with col-R AUGC at 0 mg/L colistin; [d] — p < 0.001 for comparison with col-R AUGC at 0 mg/L colistin; [e] — p > 0.05 for comparison with col-R AUGC for isolates with disrupted *mgrB*; [f] — n = 26; [g] — n = 11; [h] — n = 15; [i] — p = 0.283 for comparison with CI of the isolates with disrupted *mgrB*.

Table 3. The competition index of col-R and col-S isolates of carba-R *K. pneumoniae* representing the same sequence types

Col-R isolates		Mechanism of colistin resistance	CFU count (SD)		CI (SD)
ST	Isolate ID		Col-R (LB agar + colistin, 10 mg/L)	Col-S + col-R (LB agar)	
ST23	37261	Unknown	80	141	1.3
	69–77	Mutant *mgrB*	39	168	0.3
	37243	Unknown	25	112	0.29
	37224	Unknown	5	114	0.05
Total ST23:			**37 (32)**	**134 (26)**	**0.48 (0.56)**
ST395	52–1659	Mutant *mgrB*	88	135	1.87
	78–1127	Unknown	3	138	0.02
	59–397	Unknown	110	153	2.5
	4469	Mutant *mgrB*	17	141	0.14
Total ST395:			**55 (53)**	**142 (8)**	**1.1 (1.24)**
ST377	76–1648	Unknown	90	335	0.37
	76–2053	Unknown	38	282	0.16
	76–2089	Mutant *mgrB*	79	232	0.52
Total ST377:			**69 (27)**	**283 (52)**	**0.35 (0.18)**
ST307	64–574	Mutant *mgrB*	33	287	0.13
	56–566	Mutant *mgrB*	68	210	0.48
	71–1375	Mutant *mgrB*	63	196	0.47
Total ST307:			**55 (19)**	**231 (49)**	**0.36 (0.2)**
ST147	37–262	Mutant *mgrB*	3	201	0.02
ST48	58–2966	Mutant *mgrB*	8	152	0.06

Note: CI — competition index; SD — standard deviation.

susceptible isolates as anticipated (Table 2). Col-R isolates of *K. pneumoniae* demonstrated normal growth kinetics at 1 mg/L colistin but their growth slowed down at 4 and 16 mg/L colistin concentrations ($p = 0.016$ and $p < 0.001$, respectively). At 64 mg/L colistin, the growth of col-R isolates was almost completely inhibited at AUGC of 0.9 (0; 3.0) OD_{600} per 1 h (Table 2).

When comparing AUGC values between col-R isolates with disrupted and wild-type *mgrB* (Table 2), we found that the *mgrB* status only insignificantly affected the kinetics of bacterial growth regardless of colistin concentrations used.

Then, we calculated the CI for 26 col-R K. pneumonia isolates co-cultured with their carba-S/col-S counterparts in order to compare their competitive ability (Fig. 2; Table 2). The mean CI value was 0.15 (0.21); 25/26 (96%) of col-R isolates had IC < 1 ranging from 0.01 to 0.53; one isolate had CI of 1. Wild type isolates and those with disrupted *mgrB* had similar CI of 0.19 (0.26) and 0.1 (0.1), respectively ($p = 0.283$; Table 2). Thus, resistance to colistin was associated with a loss of competitive ability in the majority of analyzed col-R isolates, as compared to carba-S/col-S *K. pneumoniae* isolates, which did not depend on the *mgrB* status.

The effects of colistin resistance on bacterial fitness were additionally investigated in carba-R/col-S and carba-R/col-R pairs of *K. pneumoniae* of the same sequence types. We selected isolates of 5 most common ST (ST23, ST48, ST307, ST377 and ST395) and one rare ST (ST147); at least one isolate in this subset was colistin-sensitive (Table 3). The competitive ability of all col-R isolates belonging to types ST48, ST147, ST307 and ST377 was diminished compared to the col-S isolates of the same sequence types (CI < 1). However, the situation was different for the isolates represented by sequence types ST23 and ST395. One col-R isolate of type ST23 (CI = 1.3) and 2 col-R isolates of type ST395 (CI = 1.87 and CI = 2.5, respectively) were more fit than col-S isolates (Table 3).

DISCUSSION

The majority of carba-R isolates of *K. pneumoniae* in our collection were represented by 5 major sequence types; of them, types ST307 and ST395 made up 54% of the entire sample. A while ago, ST307 was not recognized as a dominant sequence type in Russia [20, 21], but at present, it is becoming one of the leading high-risk international sequence types [7], which is consistent with our findings.

Of all carba-R isolates analyzed in this paper, 45% were resistant to colistin. The multicenter study *MARAPHON* [2, 3] showed that the prevalence of col-R isolates in the large sample of nosocomial *K. pneumoniae* isolates was generally low, in spite of an increase from 4.5% in 2012 to 7.9% in 2014. Our data might reflect the global trend of growing antibiotic resistance, including resistance to colistin. According to a 15-year retrospective study conducted at a large hospital in Athens, the proportion of col-R *K. pneumoniae* isolates from blood cultures surged from 0% in 2002 to 26.9% in 2016 [22]. On the other hand, the high prevalence of col-R strains in our collection might be explained by the fact that our sample was dominated by nosocomial strains recovered from intensive care units, where, as reported by Feretzakis et al. [23], the proportion of col-R *K. pneumoniae* can be much higher than in other hospital departments (40 vs 13.8%). Besides, direct comparative analysis of colistin resistance studies that rely on different susceptibility testing techniques can be quite challenging. For example, false results are not rare in epsilometer tests in comparison with the reference method of microdilutions; therefore, such tests can fail in detecting the true rate of colistin resistance [9, 20].

Colistin resistance did not affect the kinetics of bacterial growth in the absence of this antibiotic and did not depend on the status of the *mgrB* gene, which is consistent with previously published data [24]. In contrast, in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* resistance to colistin

undermines the dynamics of bacterial growth [13, 25], which might explain the relatively high prevalence of enterobacteria possessing chromosomal resistance to colistin in comparison with col-R *A. baumannii* and *P. aeruginosa*.

At the same time, the majority of col-R isolates were less competitive than col-S isolates of *K. pneumoniae*; this is also typically seen in other bacteria, such as *A. baumannii* [13] and *P. aeruginosa* [25]. There are reports of reduced bacterial fitness in col-R *K. pneumoniae* that carry the *mcr-1* gene [26].

Another interesting finding came from the experiments comparing bacterial fitness between col-R and col-S *K. pneumoniae* isolates of one sequence type, i.e. bacteria with very similar genotypes but different susceptibility to colistin. Six different sequence types were analyzed. The majority of col-R isolates had low CI. At the same time, 2 col-R isolates of type ST395 and 1 col-R isolate of type ST23 were more competitive than col-S isolates of the same sequence type. This finding can be explained by the presence of compensatory mutations in the bacterial genome, as was the case with resistance to fluoroquinolones and colistin in *Escherichia coli* [27] and *A. baumannii* [28]. Unlike mutations that confer resistance, compensatory mutations boost bacterial fitness and thus promote resistance even in the absence of selective pressure exerted by an antibiotic [27, 28].

We conclude that resistance to colistin is common in the population of carba-R *K. pneumoniae* isolated from Moscow patients. This alarming trend requires close monitoring.

CONCLUSION

Our collection of carba-R *K. pneumoniae* isolates was dominated by sequence types ST307 and ST395; disruption of the *mgrB* gene by a variety of insertion sequences was the leading mechanism of colistin resistance.

Resistance to colistin did not affect the kinetics of bacterial growth in col-R *K. pneumoniae* in the absence of the antibiotic and did not depend on the status of the *mgrB* gene; the overwhelming majority of col-R *K. pneumoniae* isolates were less competitive than col-S strains; but within one sequence-type, there could be col-R isolates with increased competitive ability. Further research into bacterial fitness might elucidate the causes underlying the spread of colistin resistance among enterobacteria.

References

1. Suvorova MP, Yakovlev SV, Beloborodov VB, Basin EE, Eliseeva KV, Kovelenov SV. Rasprostranennost' i klinicheskoe znachenie nozokomial'nykh infektsiy v lechebnykh uchrezhdeniyakh Rossii: issledovanie ERGINI. Antibiotiki i khimioterapiya. 2016; 61 (5–6): 32–42. Russian.
2. Sukhorukova MV, Edelstein MV, Skleenova EY, Ivanchik NV, Mikotina AV, Dekhnich AV et al. Antimicrobial resistance of nosocomial *Enterobacteriaceae* isolates in Russia: results of multicenter epidemiological study «MARATHON» 2013–2014. CMAC. 2017; 19 (1): 49–56.
3. Sukhorukova MV, Edelstein MV, Skleenova EY, Ivanchik NV, Timokhova AV, Dekhnich AV et al. Antimicrobial resistance of nosocomial *Enterobacteriaceae* isolates in Russia: results of national multicenter surveillance study «MARATHON» 2011–2012. CMAC. 2014; 16 (4): 254–65.
4. Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: Epidemiology, genetic context, treatment options, and detection methods. Front Microbiol. 2016; 7: 1–30.
5. Wyres KL, Holt KE. *Klebsiella pneumoniae* Population Genomics and Antimicrobial-Resistant Clones. Trends Microbiol. 2016; 24 (12): 944–56.
6. Izdebski R, Baraniak A, Zabicka D, Machulska M, Urbanowicz P, Fiett J, et al. *Enterobacteriaceae* producing OXA-48-like carbapenemases in Poland, 2013-January 2017. J Antimicrob Chemother. 2018; 73 (3): 620–25.
7. Wyres KL, Hawkey J, Hetland MAK, Fostervold A, Wick RR, Judd LM, et al. Emergence and rapid global dissemination of CTX-M-15-associated *Klebsiella pneumoniae* strain ST307. J Antimicrob Chemother. 2019; 74 (3): 577–81.
8. Ah YM, Kim AJ, Lee JY. Colistin resistance in *Klebsiella pneumoniae*. International Journal of Antimicrobial Agents. 2014; 44 (1): 8–15.
9. Rojas LJ, Salim M, Cober E, Richter SS, Perez F, Salata RA et al. Colistin Resistance in Carbapenem-Resistant *Klebsiella pneumoniae*: Laboratory Detection and Impact on Mortality. Clin Infect Dis. 2017; 64 (6): 711–8.
10. Poirel L, Aurelie J, Nordmann P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. Clin Microbiol Rev. 2017; 30 (2): 557–96.
11. Guo B, Abdelraouf K, Ledesma KR, Nikolaou M, Tam VH. Predicting bacterial fitness cost associated with drug resistance. J Antimicrob Chemother. 2012; 67 (4): 928–32.
12. Ternent L, Dyson RJ, Krachler AM, Jabbari S. Bacterial fitness shapes the population dynamics of antibiotic-resistant and -susceptible bacteria in a model of combined antibiotic and anti-virulence treatment. J Theor Biol. 2015; 372: 1–11.
13. Beceiro A, Moreno A, Fernandez N, Vallejo JA, Aranda J, Adler B, et al. Biological cost of different mechanisms of colistin resistance and their impact on virulence in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2014; 58 (1): 518–26.
14. Choi MJ, Ko KS. Loss of hypermucoviscosity and increased fitness cost in colistin-resistant *Klebsiella pneumoniae* sequence type 23 strains. Antimicrob Agents Chemother. 2015; 59 (11): 6763–73.
15. eucast.org [Internet]. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Break- point tables for interpretation of MICs and zone diameters, version 8.0; c2018 [cited 2018 Dec 25]. Available from: http://www.eucast.org/clinical_breakpoints/.
16. Shamina OV, Kryzhanovskaya OA, Lazareva AV, Alyabieva NM, Polikarpova SV, Karaseva OV, et al. Emergence of a ST307 clone carrying a novel insertion element MITEKpn1 in the *mgrB* gene among carbapenem-resistant *Klebsiella pneumoniae* from Moscow, Russia. Int J Antimicrob Agents. 2020; 55 (2): 105850.
17. Klebsiella pneumoniae MLST [baza dannyh]. Available from: http://www.pasteur.fr/mlst/. Russian.
18. ISfinder database [Internet] [cited 2018 Nov 18]. Available from: http://www-is.biotoul.fr/is.html.
19. Baron S, Hadjadj L, Rolain JM, Olaitan AO. Molecular mechanisms of polymyxin resistance: knowns and unknowns. Int J Antimicrob Agents. 2016; 48 (6): 583–91.
20. Shamina OV, Kryzhanovskaya OA, Lazareva AV, Polikarpova SV, Karaseva OV, Chebotar IV et al. The comparison of methods for determination of colistin susceptibility in carbapenem-resistant *Klebsiella pneumoniae*. Klin Lab Diagn. 2018; 63 (10): 646–50.
21. Ageevets VA. Molekulyarnaya kharakteristika produtsentov karbapenemaz semeystva Enterobacteriaceae, vydelennykh v Sankt-Peterburge [dissertatsiya]. Spb., 2016. Russian.
22. Tansarli GS, Papaparaskevas J, Balaska M, Samarkos M, Pantazatou A, Markogiannakis A, et al. Colistin resistance in carbapenemase-producing *Klebsiella pneumoniae* bloodstream isolates: Evolution over 15 years and temporal association with colistin use by time series analysis. Int J Antimicrob Agents. 2018; 52 (3): 397–403.
23. Feretzakis G, Loupelis E, Sakagianni A, Skarmoutsou N, Michelidou S, Velentza A, et al. A 2-year single-centre audit on

antibiotic resistance of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains from an intensive care unit and other wards in a general public hospital in Greece. Antibiotics (Basel). 2019; 8 (2): 62.
24. Cannatelli A, Santos-Lopez A, Giani T, Gonzalez-Zorn B, Rossolini GM. Polymyxin resistance caused by *mgrB* inactivation is not associated with significant biological cost in *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2015; 59 (5): 2898–900.
25. Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, et al. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother. 2012; 56 (2): 1019–30.
26. Nang SC, Morris FC, McDonald MJ, Han ML, Wang J, Strugnell RA, et al. Fitness cost of *mcr-1*-mediated polymyxin resistance in *Klebsiella pneumoniae*. J Antimicrob Chemother. 2018; 73 (6): 1604–10.
27. Marcusson LL, Frimodt-Møller N, Hughes D. Interplay in the selection of fluoroquinolone resistance and bacterial fitness. PLoS Pathog. 2009; 5 (8): e1000541.
28. Mu X, Wang N, Li X, Shi K, Zhou Z, Yu Y, et al. The Effect of Colistin Resistance-Associated Mutations on the Fitness of *Acinetobacter baumannii*. Front Microbiol. 2016; 7: 1715.

Литература

1. Суворова М. П., Яковлев С. В., Белобородов В. Б., Басин Е. Е., Елисеева К. В., Ковеленов С. В. Распространенность и клиническое значение нозокомиальных инфекций в лечебных учреждениях России: исследование ЭРГИНИ. Антибиотики и химиотерапия. 2016; 61 (5–6): 32–42.
2. Сухорукова М. В., Эйдельштейн М. В., Склеенова Е. Ю., Иванчик Н. В., Микотина А. В., Дехнич А. В. и др. Антибиотикорезистентность нозокомиальных штаммов *Enterobacteriaceae* в стационарах России: результаты многоцентрового эпидемиологического исследования «МАРАФОН» 2013–2014. Клиническая микробиология и антимикробная химиотерапия. 2017; 19 (1): 49–56.
3. Сухорукова М. В., Эйдельштейн М. В., Склеенова Е. Ю., Иванчик Н. В., Тимохова А. В., Дехнич А. В. и др. Антибиотикорезистентность нозокомиальных штаммов *Enterobacteriaceae* в стационарах России: результаты многоцентрового эпидемиологического исследования МАРАФОН в 2011–2012 гг. Клиническая микробиология и антимикробная химиотерапия. 2014; 16 (4): 254–65.
4. Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: Epidemiology, genetic context, treatment options, and detection methods. Front Microbiol. 2016; 7: 1–30.
5. Wyres KL, Holt KE. *Klebsiella pneumoniae* Population Genomics and Antimicrobial-Resistant Clones. Trends Microbiol. 2016; 24 (12): 944–56.
6. Izdebski R, Baraniak A, Zabicka D, Machulska M, Urbanowicz P, Fiett J, et al. *Enterobacteriaceae* producing OXA-48-like carbapenemases in Poland, 2013-January 2017. J Antimicrob Chemother. 2018; 73 (3): 620–25.
7. Wyres KL, Hawkey J, Hetland MAK, Fostervold A, Wick RR, Judd LM, et al. Emergence and rapid global dissemination of CTX-M-15-associated *Klebsiella pneumoniae* strain ST307. J Antimicrob Chemother. 2019; 74 (3): 577–81.
8. Ah YM, Kim AJ, Lee JY. Colistin resistance in *Klebsiella pneumoniae*. International Journal of Antimicrobial Agents. 2014; 44 (1): 8–15.
9. Rojas LJ, Salim M, Cober E, Richter SS, Perez F, Salata RA et al. Colistin Resistance in Carbapenem-Resistant *Klebsiella pneumoniae*: Laboratory Detection and Impact on Mortality. Clin Infect Dis. 2017; 64 (6): 711–8.
10. Poirel L, Aurelie J, Nordmann P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. Clin Microbiol Rev. 2017; 30 (2): 557–96.
11. Guo B, Abdelraouf K, Ledesma KR, Nikolaou M, Tam VH. Predicting bacterial fitness cost associated with drug resistance. J Antimicrob Chemother. 2012; 67 (4): 928–32.
12. Ternent L, Dyson RJ, Krachler AM, Jabbari S. Bacterial fitness shapes the population dynamics of antibiotic-resistant and -susceptible bacteria in a model of combined antibiotic and anti-virulence treatment. J Theor Biol. 2015; 372: 1–11.
13. Beceiro A, Moreno A, Fernandez N, Vallejo JA, Aranda J, Adler B, et al. Biological cost of different mechanisms of colistin resistance and their impact on virulence in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2014; 58 (1): 518–26.
14. Choi MJ, Ko KS. Loss of hypermucoviscosity and increased fitness cost in colistin-resistant *Klebsiella pneumoniae* sequence type 23 strains. Antimicrob Agents Chemother. 2015; 59 (11): 6763–73.
15. eucast.org [Internet]. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Break- point tables for interpretation of MICs and zone diameters, version 8.0; c2018 [cited 2018 Dec 25]. Available from: http://www.eucast.org/clinical_breakpoints/.
16. Shamina OV, Kryzhanovskaya OA, Lazareva AV, Alyabieva NM, Polikarpova SV, Karaseva OV, et al. Emergence of a ST307 clone carrying a novel insertion element MITEKpn1 in the *mgrB* gene among carbapenem-resistant *Klebsiella pneumoniae* from Moscow, Russia. Int J Antimicrob Agents. 2020; 55 (2): 105850.
17. *Klebsiella pneumoniae* MLST [база данных]. Доступно по ссылке: http://www.pasteur.fr/mlst/.
18. ISfinder database [Internet] [cited 2018 Nov 18]. Available from: http://www-is.biotoul.fr/is.html.
19. Baron S, Hadjadj L, Rolain JM, Olaitan AO. Molecular mechanisms of polymyxin resistance: knowns and unknowns. Int J Antimicrob Agents. 2016; 48 (6): 583–91.
20. Шамина О. В., Крыжановская О. А., Лазарева А. В., Поликарпова С. В., Карасёва О. В., Чеботарь И. В. и др. Сравнение методов определения устойчивости к колистину у карбапенемрезистентных штаммов *Klebsiella pneumoniae*. Клиническая лабораторная диагностика. 2018; 63 (10): 646–50.
21. Агеевец В. А. Молекулярная характеристика продуцентов карбапенемаз семейства *Enterobacteriaceae,* выделенных в Санкт-Петербурге [диссертация]. Спб., 2016.
22. Tansarli GS, Papaparaskevas J, Balaska M, Samarkos M, Pantazatou A, Markogiannakis A, et al. Colistin resistance in carbapenemase-producing *Klebsiella pneumoniae* bloodstream isolates: Evolution over 15 years and temporal association with colistin use by time series analysis. Int J Antimicrob Agents. 2018; 52 (3): 397–403.
23. Feretzakis G, Loupelis E, Sakagianni A, Skarmoutsou N, Michelidou S, Velentza A, et al. A 2-year single-centre audit on antibiotic resistance of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains from an intensive care unit and other wards in a general public hospital in Greece. Antibiotics (Basel). 2019; 8 (2): 62.
24. Cannatelli A, Santos-Lopez A, Giani T, Gonzalez-Zorn B, Rossolini GM. Polymyxin resistance caused by *mgrB* inactivation is not associated with significant biological cost in *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2015; 59 (5): 2898–900.
25. Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, et al. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother. 2012; 56 (2): 1019–30.
26. Nang SC, Morris FC, McDonald MJ, Han ML, Wang J, Strugnell RA, et al. Fitness cost of *mcr-1*-mediated polymyxin resistance in *Klebsiella pneumoniae*. J Antimicrob Chemother. 2018; 73 (6): 1604–10.
27. Marcusson LL, Frimodt-Møller N, Hughes D. Interplay in the selection of fluoroquinolone resistance and bacterial fitness. PLoS Pathog. 2009; 5 (8): e1000541.
28. Mu X, Wang N, Li X, Shi K, Zhou Z, Yu Y, et al. The Effect of Colistin Resistance-Associated Mutations on the Fitness of *Acinetobacter baumannii*. Front Microbiol. 2016; 7: 1715.