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RESEARCH ARTICLE

Detection of different colistin resistance mechanisms among multidrug resistant *Klebsiella pneumoniae* isolates in Bulgaria

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ABSTRACT

The more frequent usage of colistin resulted in an increase of colistin resistance due to lipopolysaccharide modifications. The aim of this study was to reveal the prevalence and mechanisms of colistin resistance among multidrug-resistant *Klebsiella pneumoniae* isolates collected in Bulgaria. One hundred multidrug resistant *K. pneumoniae* isolates were collected in a period between 2017 and 2018. Among them, 29 colistin resistant and 8 heteroresistant isolates were observed and further investigated. Clonal relatedness was detected by RAPD and MLST. Carbapenemases, two component system *phoQ/phoP*, *pmrA/B*, and *mgrB* were investigated by PCR amplification and Sanger sequencing. Among 37 colistin nonsusceptible isolates, we detected 25 NDM-1 producers. The isolates belonged mainly to ST11 (80%), and also to ST147, ST35, ST340, ST219 (1-2 members per clone). Nine colistin resistant isolates showed changes in *mgrB*. IS903B-like elements truncated *mgrB* in five isolates. In two isolates, premature stopcodon (Q30stopcodon) was observed and another two isolates did not amplify *mgrB*, possibly due to bigger deletion or insertion. No isolates showed *pho*Q/*pho*P and *pmr*A/B mutations except for *pmrB* (four isolates had R256G). All isolates with IS903B insertions belonged to ST11 clone. The *mgrB* alterations play major role in colistin resistance in *K. pneumoniae* isolates studied in the current work. We report truncation of *mgrB* by IS903 like element in colistin resistant NDM-1 producing *K. pneumoniae* ST11 clone in Bulgaria.

KEYWORDS

Bulgaria, *mgrB*, colistin resistance, *K pneumoniae*, IS903

INTRODUCTION

Klebsiella pneumoniae causes serious hospital acquired infections (respiratory, urinary, soft tissue infection and invasive infections as sepsis) and it can acquire many resistance determinants by transfer of plasmids, transposons/integrons or by mutations (1). The most important mechanisms of resistance include extended-spectrum beta-lactamases (ESBLs). The ESBLs historically include two main families namely, SHV and TEM, which develop from SHV-1 and TEM-1 enzymes through point mutations. During the last decades, CTX-M group has become the main ESBL family. The main beta-lactamases in this group are CTX-M-15 and CTX-M-3 that became dominant all over the world [1, 2]. ESBL producing isolates are commonly resistant to beta-lactams and to other antibiotic classes such as aminoglycosides and fluoroquinolones, and leave only carbapenems as a possible therapy [1]. On the

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basis of increasing usage of carbapenems, the numbers of carbapenemases detected in *Klebsiella* spp. also increased. Serin active carbapenemases KPC and OXA-48 as well as metallo-beta-lactamase NDM have been reported to be associated with *Klebsiella* spp. all over the world [3, 4]. The emergence and rapid spread of carbapenemase-producing strains not only among the *Klebsiella* spp., but also among other *Enterobacterales*, *Acinetobacter* spp. and *Pseudomonas aeruginosa*, which could became resistant to almost all antibiotics available, have led to renewal of polymyxin use in medicine. WHO has included colistin in its list of critically important antibiotics [5]. The increased and inappropriate colistin use was followed by rapid appearance of lipopolysaccharide (LPS) modifications. Colistin resistance involves changes in LPS structure with addition of 4-amino-4-deoxi-L-arabinose or phosphoethanolamine. These modifications occur in *pmrCAB* and *arnBCADTEF-pmrE* operons and thus, increase the positive charge on LPS and therefore, decrease colistin binding, leading to colistin resistance [6]. These mechanisms are under control of the two component systems PhoP/PhoQ and PmrA/PmrB. Mutation in the genes encoding regulatory systems could result in their constitutive over expression, which will activate *pmrCAB* and *arnBCADTEF-pmrE* systems. In addition, PhoP/PhoQ system is subjected to negative feedback regulation by MgrB protein [6, 7]. In recent years, plasmid-mediated colistin resistance encoded by *mcr* genes has also been reported [8]. The *mcr* genes encoding phosphoethanolamine transferase add phosphoethanolamine and decrease negative charge of LPS thus, decrease LPS affinity to colistin [6, 7]. Since their first identification in the late 2015 [8], various new alleles of transferable colistin resistance determinants have detected [9].

In Bulgaria, the level of third generation cephalosporin resistant invasive *K. pneumoniae* isolates was very high, above 79.1% in 2020 [10]. In addition, we had an increased level of carbapenemase-producing *K. pneumoniae* isolates as well [11]. The prevalence of carbapenem resistant invasive *K. pneumoniae* isolates was 28.1% for 2020 versus 4.4% for 2016 [10]. So far, the mechanisms of colistin resistance in Bulgaria has not been investigated. The aim of this study was to reveal the frequency and mechanisms of chromosomal or plasmid-mediated colistin resistance in a collection of multidrug resistant *K. pneumoniae* isolates collected in Bulgaria in 2017–2018.

MATERIAL AND METHODS

Bacterial strains

In the period January 2017 – May 2018, multidrug-resistant (resistant to three and more groups of antimicrobial agents, according to Magiorakos et al. [12]) *K. pneumoniae* isolates were collected. The strains were isolated from specimens of patients in Alexandrovska University Hospital in Sofia, Bulgaria. The isolates were obtained from urine (n = 65, 65%), respiratory samples (n = 19, 19%), wounds and soft tissue samples (n = 8, 8%), blood cultures (n = 4, 4%), abdominal fluid (n = 1, 1%), stool (n = 2, 2%) and central venous catheter (n = 1, 1%). The species identification was done by BBL Crystal E/NF identification system (BD) or by Vitek MS (bioMerieux S.A., France) according to the manufacturer's instructions.

Susceptibility testing

Antimicrobial susceptibility testing to all antibiotics, except for colistin and tigecycline was performed by disk-diffusion method, according to EUCAST guidelines [13]. Interpretation of the results was done according to EUCAST breakpoints [13]. Colistin minimum inhibitory concentrations (MICs) were determined by broth microdilution method (BMD) in cation-adjusted Mueller-Hinton II broth (MHB II, BioLab ZRt) with two-fold dilutions of colistin sulfate (Sigma-Aldrich), according to the current joint CLSI-EUCAST working group recommendations and the ISO standard 20776-1 [14]. *E. coli* ATCC 25922 and *E. coli* NCTC 13846 were used as quality control strains. In addition to BMD, the Superpolymixin medium (SPM) for detection of colistin resistant isolates was used [15]. Tigecycline susceptibility was evaluated by MIC strip method (Liofilchem, Italy).

Phenotypic detection of carbapenemases

Phenotypic detection of carbapenemase production in investigated *K. pneumoniae* isolates was performed by metallo-beta-lactamase (MBL), KPC (*K. pneumoniae* carbapenemase) and OXA (Oxacillinase carbapenemase)-48 Confirm kit (Rosco diagnostica A/S, Denmark), according to the manufacturer's instructions.

Molecular identification of ESBL and carbapenemase genes

All isolates were PCR screened for the presence of bla_{VIM}, bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48,}}$ and $bla_{\text{CTX-M}}$ as previously described [16, 17] as well as of bla_{CMY}, bla_{FOX}, bla_{DHA}, and bla_{AAC} [18].

Genes were sequenced using primers binding outside the coding region of $bla_{\text{CTX-M-1-group}}$, bla_{CMY} [11] and bla_{NDM} [19]. Nucleotide and deduced amino acid sequences were analysed and multiple alignments were performed using Chromas Lite 2.01 (Technelysium Pty Ltd, Brisbane, Australia) and DNAMAN version 8.0 Software (Lynnon BioSoft, Vaudreuil-Dorion, Canada).

Clonality analyis

Total bacterial DNA was prepared using DNA SorbA kit (*Sacace Biotechnologies, Italy*). ERIC PCR was performed as previously described [17]. Genetic similarity was determined using Dice coefficient as similarity measure and the unweighted pair group method with arithmetic mean (UPGMA) (http://genomes.urv.cat/UPGMA/). A clone was defined as isolates showing 80% similarity.

For the *K. pneumoniae* isolates, Multilocus Sequence Typing (MLST) based on seven conserved housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB* and *tonB*) and assignment to allelic numbers and sequence-types (STs) were performed as described in the MLST database (Pasteur Institute, Paris, France; http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html). A clonal complex was defined as a group of two or more independent isolates that share six identical alleles.

Molecular identification of mechanisms of colistin resistance

Chromosomal *phoP/phoQ*, *pmrA/pmrB* and *mgrB* genes associated with colistin resistance were amplified with primer sets as described previously [7, 20]. The amplicons were sequenced after treating with Rapid PCR Cleanup Enzyme Set (ExoSAP, Applied Biosystems,USA). The sequencing was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit и BigDye® Terminator v1.1 & v3.1 5X Sequencing Buffer, on the sequencer model Applied Biosystems 3130xl Genetic Analyzer. The analysis was made with Chromas Lite version 2.01 (Technelysium Pty Ltd, Australia), DNAman version 8 (Lynnon Corporation, USA) and BLAST program (http://www.ncbi.nlm.nih.gov). Two colistin susceptible isolates were investigated as control isolates. The type of insertion sequence was observed with ISFinder (https://www-is.biotoul.fr) [21].

Plasmid mediated colistin resistance determinants (*mcr-1, mcr-2, mcr-3, mcr-4, mcr-5*) were investigated with multiplex PCR suggested by Lescat et al. [22].

RESULTS

One hundred multidrug resistant *K. pneumoniae* isolates were collected from January 2017 to May 2018. All isolates were multidrug resistant, the susceptibility is shown on Table 1. Most of the isolates were carbapenem nonsusceptible (65%, 65/100). Colistin BMD had revealed 29 (29%) isolates as colistin resistant with MICs ranging between 4 and 128 mg L^{-1}, MIC_{50} of 32 mg L^{-1} and MIC_{90} of 64 mg L^{-1}. On SPM media grew 37 isolates - 29 detected by BMD as colistin resistant and 8 isolates were colistin susceptible according BMD. We classified these 8 isolates as heteroresistant.

Only colistin resistant and heteroresistant isolates were further investigated. The susceptibility and characteristics of these isolates are shown on Table 2. The colistin resistant/heteroresistant isolates were susceptible mainly to imipenem (32%, 12/37), meropenem (21.6%, 8/37), gentamicin (30%,11/37), tigecycline (36%, 12/33) and amikacin (19%,7/37). Only eight isolates were imipenem and meropenem susceptible. Most of colistin resistant/heteroresistant isolates were carbapenemase producers and ROSCO disks showed that 25 of them were metallo-beta-lactamase producers. PCR reactions confirmed production of carbapenemases in 25 of the isolates and they showed presence of NDM enzyme. Most (18 isolates) of NDM producing isolates produced CMY enzymes as well. The sequences revealed presence of bla_{NDM-1} and bla_{CMY-4}. Most of isolates were bla_{CTX-M} positive (32 isolates, including 12 carbapenem susceptible isolates) and sequence showed presence of $bla_{CTX-M-15}$, and two carbapenem nonsusceptible and carbapenemase negative isolates were $bla_{CTX-M-15}$ and bla_{CMY-4} positive.

Clonality analysis showed that majority of the isolates belonged to the main clone P (30 isolates – 81%), the other clones were with small numbers (F in two isolates, B in two isolates, and C and K in one isolate each).

MLST showed that members of the clone P belonged to ST11, members of clone B to ST 147, members of clone C – ST35, those of clone K to ST340, and those of clone F to ST219.

Table 1. Antimicrobial susceptibility of 100 multidrug resistant *K. pneumoniae* isolates

Antimicrobial agent	Tested isolates	S	I	R
Amoxicillin/clavulanic acid	100	0	3 (3%)	97 (97%)
Piperacillin/tazobactam	100	6 (6%)	3 (3%)	91 (91%)
Cefuroxime	100	0	0	100 (100%)
Cefoxitine	100	16 (16%)	0	84 (84%)
Cefotaxime	100	0	0	100 (100%)
Ceftriaxone	100	0	0	100 (100%)
Ceftazidime	100	0	0	100 (100%)
Cefepime	100	1 (1%)	0	99 (99%)
Imipenem	100	35 (35%)	11 (11%)	54 (54%)
Meropenem	100	28 (28%)	4 (4%)	68 (68%)
Gentamicin	100	22 (22%)	30 (30%)	48 (48%)
Amikacin	100	25 (25%)	8 (8%)	67 (67%)
Tobramycin	100	9 (9%)	0	91 (91%)
Ciprofloxacin	100	5 (5%)	0	95 (95%)
Trimethoprime/sulfamethoxazole	100	10 (10%)	0	90 (90%)
Chloramphenicol*	100	26 (26%)	0	74 (74%)
Tigecycline	87	29 (33.33%)	19 (21.84%)	10 (11.49%)
Colistin	100	29 (29%)	–	71 (71%)

Abbreviations: S- susceptible, R-resistant, I-intermidiate.

Table 2. Characterictics of 37 colistin nonsuceptible *K. pneumoniae* isolates

Number	Year	specimen	MEM	IMI	Carbape nemase	Colistin MICs, mg L^{-1}			The isolate was susceptible to:	Chromosomal genes mutations						ERIC	MLST
						Interp	MIC_1	MIC_2		*mgrB*	*pmrA*	*pmrB*	*phoP*	*phoQ*	*mcr*		
2K	2017	ur	R	R	NDM-1	R	>128		–	*IS903 like*	intact	intact	intact	intact	neg	P	11
11K	2017	eta	R	I	NDM-1	hR	msw	4	TGC	intact	intact	intact	intact	intact	neg	P	
12K	2017	wd	R	R	NDM-1	hR	1	32	–	intact	intact	intact	intact	intact	neg	P	11
14K	2017	ur	S	S	–	R	>128		IMI, MEM, AN, TZP,	intact	intact	intact	intact	intact	neg	p	11
15K	2017	ur	S	S	–	hR	0.5	32	IMI, MEM, GM, AN, NN, FOX, TGC	intact	intact	intact	intact	intact	neg	K	340
24K	2017	eta	R	R	NDM-1	hR	msw		–	intact	intact	intact	intact	intact	neg	P	
30K	2017	ur	S	S	–	hR	0.5	32	IMI, MEM, GM, AN, FOX, TZP, TGC, CHL	intact	intact	R256G	intact	intact	neg	c	35
32K	2017	ur	R	R	NDM-1	R	64		GM	(–)*	intact	intact	intact	intact	neg	P	
41K	2017	ur	R	S	–	R	32		IMI, GM	intact	intact	intact	intact	intact	neg	P	11
53K	2017	eta	S	S	–	hR	1	16	IMI, MEM, GM, AN, FOX	intact	intact	intact	intact	intact	neg	B	147
57K	2017	ur	R	R	NDM-1	R	16		–	intact	intact	intact	intact	intact	neg	P	
59K	2017	ur	I	S	–	R	32		IMI, GM	intact	intact	intact	intact	intact	neg	P	11
61K	2017	ur	R	R	NDM-1	R	8		TGC	intact	intact	intact	intact	intact	neg	P	
67K	2017	ur	S	S	–	R	64		IMI, MEM, GM, AN, NN	stop codon	intact	R256G	intact	intact	neg	F	219
68K	2017	ur	R	R	NDM-1	R	16		–	intact	intact	intact	intact	intact	neg	P	
69K	2017	ps	S	S	–	R	64		IMI, MEM, GM, AN, NN	stop codon	intact	R256G	intact	intact	neg	F	219
70K	2017	ca	R	R	NDM-1	R	64		–	intact	intact	intact	intact	intact	neg	P	
72K	2017	wd	S	S	–	R	64		IMI, MEM	intact	intact	R256G	intact	intact	neg	c	35
73K	2017	eta	R	R	NDM-1	R	8		–	intact	intact	intact	intact	intact	neg	P	
74K	2017	ur	S	S		hR	0.5	64	GM, IMI	intact	intact	intact	intact	intact	neg	P	11
77K	2017	ur	R	S	–	R	64		IMI	intact	intact	intact	intact	intact	neg	P	
83K	2017	ur	R	R	NDM-1	R	16		GM, TGC	intact	intact	intact	intact	intact	neg	P	11
87K	2017	ur	R	S	NDM-1	R	64		IMI, TGC	intact	intact	intact	intact	intact	neg	P	
88K	2017	ur	R	R	NDM-1	R	16		TGC	intact	intact	intact	intact	intact	neg	P	11
90K	2018	ur	R	R	NDM-1	R	16		GM	intact	intact	intact	intact	intact	neg	P	
91K	2018	eta	R	I	NDM-1	R	128		TGC	*IS903B like*	intact	intact	intact	intact	neg	P	11
94K	2018	ur	R	R	NDM-1	R	32		TGC	(–)*	intact	intact	intact	intact	neg	P	
97K	2018	st	R	I	–	hR	0.25	128	SXT	intact	intact	intact	intact	intact	neg	B	147
100K	2018	wd	R	R	NDM-1	R	64		–	*IS903B like*	intact	intact	intact	intact	neg	P	11
104K	2018	ur	R	R	NDM-1	R	4		–	intact	intact	intact	intact	intact	neg	P	
108K	2018	eta	R	R	NDM-1	R	64		–	*IS903B like*	intact	intact	intact	intact	neg	P	11
109K	2018	ur	R	R	NDM-1	R	16		–	intact	intact	intact	intact	intact	neg	P	

(continued)

Table 2. Continued

Number	Year	specimen	MEM	IMI	Carbapenemase	Colistin MICs, mg L^{-1}			The isolate was susceptible to:	Chromosomal genes mutations						ERIC	MLST
						Interp	MIC_1	MIC_2		*mgrB*	*pmrA*	*pmrB*	*phoP*	*phoQ*	*mcr*		
112K	2018	ps	R	R	NDM-1	R	128		–	*IS903B like*	intact	intact	intact	intact	neg	P	11
113K	2018	ur	R	R	NDM-1	R	8		–	intact	intact	intact	intact	intact	neg	P	
117K	2018	bl	R	R	NDM-1	R	32		TGC	intact	intact	intact	intact	intact	neg	F	219
120K	2018	ur	R	R	NDM-1	R	32		TGC	intact	intact	intact	intact	intact	neg	P	
121K	2018	ur	R	R	NDM-1	R	32		–	intact	intact	intact	intact	intact	neg	P	11

Abbreviations: Colistin – colistin MICs evaluation according EUCAST 2020.
MIC1 – Colistin MICs determined with BMD.
MIC2 – Colistin MICs observed after recultivation of colonies of the resistant subpopulations, grown on SPM.
msw: mutation selection window.
*, lack of amplification; ur – urine, bl – blood, wd – wound, eta – endotracheal aspirate; ps – pus, ca – venous catheter, sp – sputum.
IMI – imipenem, MEM – meropenem, FOX – cefoxitin, GM – gentamicin, AN – amikacin, NN – tobramycin, TGC – tigecycline, TZP – piperacillin/tazobactam, SXT – trimethoprim/sulfamethoxazole, CHL – chloramphenicol.

Mechanisms of colistin resistance

The amplified and sequenced genes from the two component systems *phoP/phoQ* and *pmrA* did not show difference in comparison to colistin susceptible isolate IT977, accession number KY587108 and two colistin susceptible isolates that were used as controls. All but four isolates did not show mutations in *PmrB*. These four isolates showed an exchange of arginin/glycine R256G. One colistin susceptible isolate had the same R256G exchange.

The entire *mgrB* gene was amplified by PCR and revealed amplicons in all but two isolates.

Five isolates generated a larger size amplicon compared to that in wild-type isolates. We found amplicons with size around 1400/1500 bp. The sequencing showed that all isolates with 1500 bp amplicon size had insertion sequence (IS) with 1057 size inserted in +52 position (for 91K, 100K, 108K, 112K isolates) and for one (2K) isolate (amplicon 1400bp) insertion in +116 position (Figs 1 and 2). IS finder classified detected sequences in IS5 family and found the higher similarity with IS903B (98% similarity). The IS of the single isolate 2K was similar to the IS of the other isolates with only one nucleotide difference, but the orientation of the IS was in the opposite direction (Figs 1 and 2). Two isolates (67 and 69 K) showed exchange of glutamine with stop codon Q30stop codon (CAG changed to TAG). All other isolates lacked mutations in *mgrB* genes (intact genes, Table 2). No mutations were detected in promoter region of all tested isolates, by *MgrB* primer set, which we chose to amplify the promoter region. All colistin heteroresistant isolates lacked genetic changes. All isolates were negative for *mcr-1,-2,-3,-4,-5* genes.

DISCUSSION

During the COVID-19 pandemic, the problem of bacterial resistance has become more and more important. With increased use of antibiotics in hospitals and community we could expect to have increased levels of resistant strains including carbapenemase producers. In this study we present the evaluation of colistin resistance mechanisms among the most frequent carbapenemase producing species in Bulgaria, *K. pneumoniae* [11, 17] right before the COVID pandemic. Colistin is one of the last-choice antibiotics used to treat infections caused by carbapenemase producing Gram-negative bacteria [23]. During the period from January 2017 to May 2018, 37 colistin nonsusceptible isolates were isolated among 100 multidrug resistant isolates. Twenty-nine of them were evaluated as colistin resistant with BMD and eight isolates were categorized as colistin heteroresistant. The prevalence of colistin resistant isolates, in addition to multidrug resistance of the isolates was alarming and showed a need of strict infection control measures. This prevalence was higher (29%) than that of detected in Europe in the period 2014-2019 (3–5% colistin-resistant isolates) [24]. But this percentage was lower than those observed in Dubai (59%) [25] and London (65.8%)

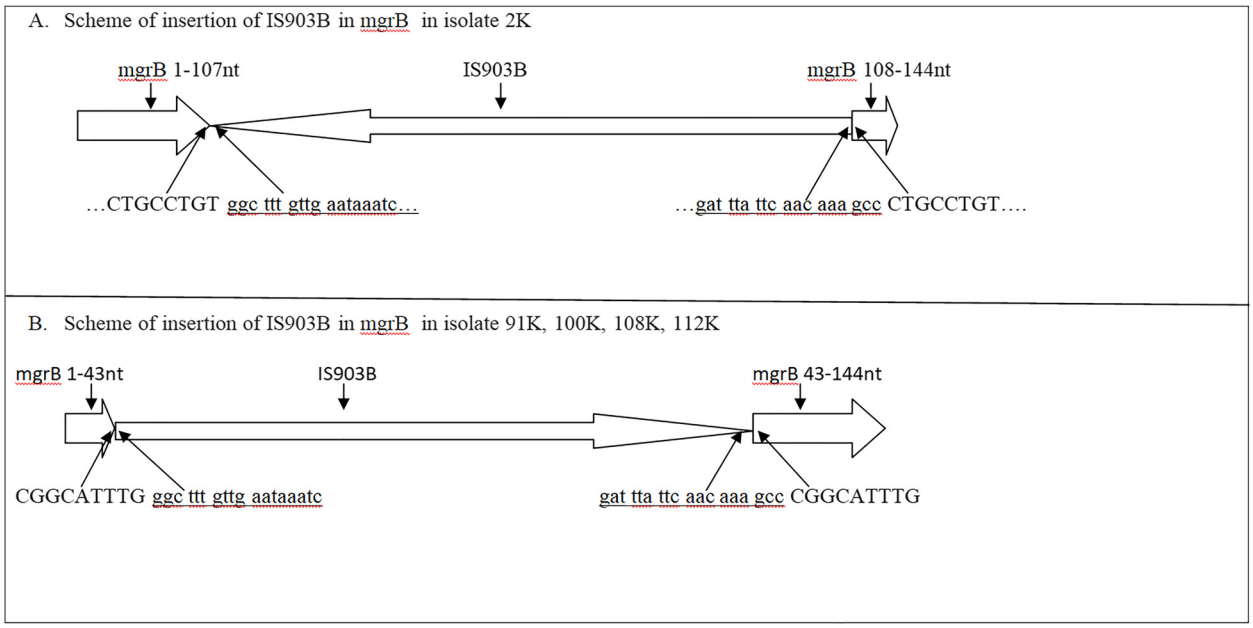

Fig. 1. Scheme of insertion of IS element in *mgr*B among multidrug resistant *K. pneumoniae* isolates
Abbreviations: Capital letters showed direct repeats and small letters/underlined showed inverted repeats

[26]. Moreover, the isolates in the present study mainly belonged (in 80%) to one clone - ST11 type. This clone is disseminated in many countries and has produced a wide spectrum of carbapenemases, mainly NDM-1 and KPC-2 [4]. Detection of NDM-1 enzyme in the present study was in concordance with previous Bulgarian report which showed an increasing number of NDM-1 producing ST11 in our country [11]. The present work revealed that this clone is further disseminated and stays stable in Bulgarian hospitals. Due to the high resistance of the isolates, we could expect increased therapeutic failures. It should also be pointed out that among colistin-resistant/heterorestant isolates, eight of them (21.6%) were susceptible to carbapenems. This phenotype is not frequently described, probably because many studies evaluated colistin susceptibility among carbapenem-resistant isolates, but it deserves high attention because these isolates are also possible to acquire resistance to carbapenems, leaving no therapeutic options for treating these infections. Microbiologists should test all isolates for colistin resistance.

Colistin heteroresistant strains produce subpopulations with higher level of polymyxin resistance than those detected by BMD. Polymyxin heteroresistant strains were more frequent among the multidrug resistant *K. pneumoniae* isolates [27]. The fact that 8 out of 100 multidrug resistant isolates were heteroresistant was alarming and showed a need of implementation of methods to detect heteroresistant isolates. The use of SPM as addition to BMD may be a good suggestion. The importance of heteroresistant isolates is pointed out in some reports which showed that infection with heteroresistant isolates led to treatment failure in a murine model [28] In our study this method successfully detected a high number heteroresistant isolates. They did not show any changes in *pmr*A/B, *pho*P/Q system or *mgrB* genes. This is in accordance with some reports revealing that heteroresistance is due to mutations in chromosomal genes, such as lipid A biosynthesis genes (*lpxA, lpxC, lpxD*) [29]. The heteroresistant isolates in this study included members of the main clone ST11 and also of the ST147, ST35, ST340.

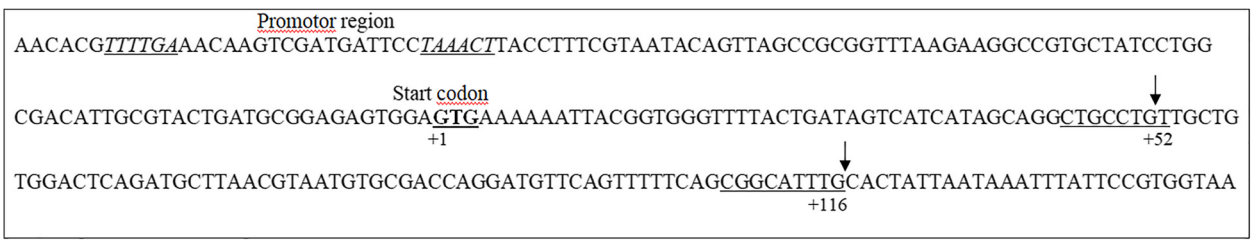

Fig. 2. The *mgrB* sequence and site of insertion of IS elements in *mgr*B among investigated *K. pneumoniae* isolates
Abbreviations: Underlined is a direct repeat and with arrow is shown site of insertion, promoter region is underlined and in italic and start codon is underlined and in bold

Investigation of colistin resistance determinants showed absence of mutations in *pmr*A/B and *pho*P/Q in almost all the colistin resistant isolates. Only four isolates showed R256G exchange in *pmrB* gene. In addition, the colistin susceptible isolate with MIC of 0.25 mg L^{-1} (data was not shown) also had such exchange. Some authors have reported that this exchange did not affect the colistin MIC of the isolates. Haeili et al. [30] found that complementation with wild type *pmrB* gene was not able to restore colistin susceptibility in isolates characterized with R256G alteration. The same findings of Cheng et al. [31] were observed by a site-directed mutagenesis method. Interestingly, our isolates did not belong to ST11 clone. For these isolates other mechanism can be supposed - two of them showed Q30stop codon.

In the present study, 9 from 29 colistin resistant isolates (31%) showed *mgrB* inactivation. For two isolates, we did not obtain any amplification. This could be due to a very large deletion or insertion in the gene. Cienfuegos-Gallet et al. [32] investigated isolates that could not amplify *mgrB* and after WGS analysis they observed a big insertion identical to plasmids in 3 of their tested strains. The results suggested a plasmid DNA integration into the chromosome.

In the present study, *mgrB* analysis revealed the presence of IS5 family insertion sequences (IS903B) which disrupt the gene in 5 colistin-resistant isolates. Inactivation of *mgrB* was often associated with colistin resistance through an increase of PhoP/Q system expression and could be due to insertions of different IS [6, 33, 34]. Insertion sequences are small mobile genetic elements and could jump to a DNA carrier or between chromosome and plasmids [35]. The truncation of *mgrB* by IS elements seems to be a key mechanism of acquired colistin resistance [36] The common IS elements that have been reported to disrupt *mgrB* were from IS5 family (IS 903, IS903B) [6, 7]. In the present study, we observed this inactivation in five colistin resistant isolates.

Other IS elements which could disrupt *mgrB* were IS*102*, IS*Kpn26*, IS*10L*, IS*Kpn13* [37]. In the present study, the hot spot of insertion were +52 and +116. These nucleotides were reported as common sites for insertions [37]. Other common regions for insertion were +15+16; +44+45; +60+61; +69+70+71; +74+77 [30, 36].

All isolates with IS insertion were member of one clone, the NDM-1 producing ST11 clone *K. pneumoniae*. The observed data showed a great variability of this clone. In previous studies in Bulgaria, ST11 clone became a rapidly dominant clone among carbapenemase producing Bulgarian *K. pneumoniae* isolates. Interestingly, the isolate 2K showed IS 903B insertion but the IS had one nucleotide substitution and the direction of IS was opposite to the direction of *mgrB*. This data emphasize the high potential of ST11 clone for genetic change. The resistance of this clone is impressive. Fourteen members (out of 37) were not susceptible to any of tested antimicrobials, including the five isolates with IS insertion. Kidd et al. [38] observed that *mgrB* inactivation not only increased colistin resistance but also augmented virulence of *K. pneumoniae*. According to Kidd et al., [38] increased virulence may be one possible explanation for the increased mortality observed in infections caused by colistin-resistant strains. Lipid A modifications resulting from *mgrB* deletion have also been found to lead to a weak and delayed inflammatory response due to impaired recognition of lipid A by TLR4/MD-2 [38].

Another explanation of colistin resistance in our isolates in the present study was mutation that caused Q30stop codon substitution. The same substitution has been found by several other studies reinforcing the hypothesis that position +88 in *mgrB* (codon 30 in protein) is a critical region [30, 33, 39]. The complementation assay performed by Haeili et al. [30] proved that reverting to the wild type genotype caused significant decrease of colistin MIC from 128 to 2 mg L^{-1}.

In 2015, the world's first report on the detection of a plasmid-encoded gene (*mcr-1*) encoding resistance to polymyxins in *Escherichia coli* emerged [8], and this was followed by numerous reports on the spread of this resistance mechanism in members of *Enterobacterales* in more than 30 countries in 5 continents [5]. In the present study, plasmid-mediated colistin resistance determinants were not observed.

In conclusion, we found that *mgrB* alterations played a major role in colistin resistance in *K. pneumoniae* isolates evaluated in the present work. Our report emphasizes the increased role of IS elements in colistin resistance in *K. pneumoniae* by *mgrB* disruption. The isolates with this resistance belonged to a highly resistant and widely disseminated clone, the NDM-1 producing *K. pneumoniae* ST11 clone.

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