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Multicentre investigation of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in Bulgarian hospitals – Interregional spread of ST11 NDM-1-producing *K. pneumoniae*

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ABSTRACT

Aim: The aim of this study was to investigate the mechanisms of beta-lactam-resistance and the clonal relatedness of carbapenem-nonsusceptible *Klebsiella pneumoniae* and *Escherichia coli* isolates, collected consecutively in eight centers in five Bulgarian cities from November 2014 to March 2018. Carbapenemase-producing enterobacteria were detected in all but one centers. Overall, 104 *K. pneumoniae* and one *E. coli* were analysed.
Materials and methods: Antimicrobial susceptibility and beta-lactamases were analysed. Conjugation experiments, plasmid fingerprinting and replicon typing, as well as MLST and ERIC-PCR were carried out.
Results: KPC-2 (51%) and NDM-1 (47%) were the main carbapenemases identified. KPC-2 producing *K. pneumoniae* were classified into 10 MLST-types. The four dominating MLST-types ST29, ST15, ST336 and ST902 comprised 79% of the KPC-2 producers. All but one of the NDM-1 producing isolates belonged to the MLST-type ST11 and were found in seven centers. Furthermore, single *K. pneumoniae* isolates producing VIM-1 (ST147) and OXA-48 (ST15) were identified. In addition to the carbapenemases, the ESBLs CTX-M-15, CTX-M-3, and SHV-12 as well as AmpC enzyme CMY-4 were found. The FIIAs-replicon-type was found in all KPC-2 producers while the A/C-replicons dominated in NDM-1 producing isolates. The single NDM-1 producing *E. coli* was determined as MLST-Type ST10 (Warwick scheme).
Conclusion: The interregional clonal expansion of NDM-1 producing ST11 *K. pneumoniae* and the dissemination of $bla_{\mathrm{KPC-2}}$ carrying plasmids were responsible for the spread of carbapenemase-producing *K. pneumoniae* in Bulgaria. Our findings highlight the urgency to prevent dissemination of these highly transmissible and dangerous lineages.

1. Introduction

The occurrence of carbapenem-resistant *Enterobacteriaceae* (CRE) clinical isolates is globally increasing (Nordmann and Poirel, 2014; Logan and Weinstein, 2017). The major mechanism of carbapenem-resistance is the production of different carbapenemases (Nordmann and Poirel, 2014; Lee et al., 2016). Their encoding genes are usually located on mobile genetic structures, like plasmids, transposons and

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integrons, together with genes for resistance to other classes of antibiotics (Nordmann and Poirel, 2014; Lee et al., 2016). Carbapenemase-producing enterobacteria (CPE) are often resistant to all or almost all antimicrobial agents in clinical use, posing a serious therapeutic problem (Tzouvelekis et al., 2014). The most common carbapenemases in *Enterobacteriaceae* are variants of the class A enzymes KPC and GES, the class D OXA-48, and the metallo-beta-lactamases (MBLs) NDM, IMP, and VIM (Lee et al., 2016; Pitout et al., 2015; Nordmann and Poirel, 2014).

Various geographic regions showed great differences in the spectrum of carbapenem-hydrolysing enzymes, detected in enteric bacteria (Nordmann and Poirel, 2014; Logan and Weinstein, 2017).

KPC-enzymes are the most common carbapenemases identified in *Klebsiella pneumoniae* isolates in many countries (Nordmann and Poirel, 2014; Pitout et al., 2015). After their first detection in 2000 in USA, KPC-producing *K. pneumoniae* have been globally spread. In some regions in USA, Latin America, Asia (Israel and China) or Europe they became endemic (Lee et al., 2016).

OXA-48 was firstly detected in a clinical isolate of *K. pneumoniae* in Turkey in 2001, and its occurrence was restricted to this country for several years. However, since 2008 its distribution, mostly in the Middle East and North African countries, has been reported. In addition to the sporadic cases, outbreaks have been registered in Belgium, France, Greece and the Netherlands (Potron et al., 2013).

NDM-producing isolates appeared in 2008 and spread rapidly all over the world (Asia, Africa, Australia, America, and Europe) (Nordmann and Poirel, 2014; Lee et al., 2016). The main reservoir of NDM-producers is the Indian subcontinent (India, Pakistan, and Sri Lanka), but the Balkan countries and the Arabic peninsula are also considered as an important source of NDM-carbapenemases (Nordmann and Poirel, 2014).

In Bulgaria, the incidence of carbapenemase-producing *Enterobacteriaceae* was very low until 2014 when reports are showing a steady increase. The both NDM-1 and KPC-2 carbapenemases were previously detected in *Escherichia coli* isolates (Poirel et al., 2014; Markovska et al., 2017b). Similarly, NDM-1 and KPC-2 producing *K. pneumoniae* isolates were reported in single cases (Todorova et al., 2016; Kostyanev et al., 2016) or outbreaks (Markovska et al., 2015; Savov et al., 2018). In 2012–2015 inter-hospital dissemination of a multidrug-resistant VIM-1 and CMY-99 producing *Proteus mirabilis* clone, affecting seven Bulgarian hospitals was detected (Markovska et al., 2017a).

The aim of this study was to investigate the mechanisms of beta-lactam resistance and the clonal relatedness of carbapenem-non-susceptible *K. pneumoniae* and *E. coli* isolated from patients, hospitalized in eight hospitals in five Bulgarian towns.

2. Material and methods

2.1. Study design and isolate collection

Between November 2014 and March 2018, eight hospital centres in five major Bulgarian cities from different parts of the country participated in the study [Fig. 1 and Table 1]. During the study period, each hospital consecutively collected non-duplicate carbapenem non-susceptible isolates of *K. pneumoniae* and *E. coli* from clinical specimens. No data concerning the transfer of patients between hospitals was available.

Species identification was performed by routine biochemical identification or API 20 E (bioMérieux, Marcy l'Etoile, France) or VITEK (bioMérieux, USA) or Phoenix (Becton Dickinson, USA).

2.2. Phenotypic confirmation of carbapenemase production and antimicrobial susceptibility testing

Fig. 1. Bulgarian cities where participating centres are situated.
The towns, where carbapenemase-producing isolates have been detected are encircled.

The phenotypic confirmation of carbapenemase production was performed by the KPC/Metallo-beta-lactamase and OXA-48 Confirm Kit (ROSCO Diagnostica, Denmark).

Antimicrobial susceptibility of the isolates was assessed by disk diffusion method. For imipenem, meropenem, and tigecycline minimal inhibitory concentrations (MICs) were determined by MIC test strips (Liofilchem, Italy), while for colistin the broth microdilution method (SensiTest Colistin, Liofilchem, Italy) was used. The results were interpreted according to the EUCAST, 2017 guidelines (http://www.eucast.org/clinical_breakpoints).

2.3. Molecular characterisation of the resistance mechanisms

All isolates were screened by PCR for the presence of *bla*$_{\text{VIM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$,*bla*$_{\text{CTX-M}}$, and *bla*$_{\text{SHV}}$ as previously described (Poirel et al., 2011; Markovska et al., 2015) as well as of *bla*$_{\text{CMY}}$, *bla*$_{\text{FOX}}$, *bla*$_{\text{DHA}}$, and *bla*$_{\text{AAC}}$ (Pérez-Pérez and Hanson, 2002). The isolates were also screened for the presence of *bla*$_{\text{OXA-III group}}$ using the following primers: OXA-1-V:CCGGAGCCTCATTAATTG; OXA-1-R:AGGGGAGCCAAAAGCTTG.

The genes were sequenced using primers binding outside the coding region of *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M-1-group}}$, *bla*$_{\text{KPC}}$ (Markovska et al., 2015), *bla*$_{\text{CMY}}$, *bla*$_{\text{VIM}}$ (Markovska et al., 2017a) and *bla*$_{\text{NDM}}$ (Rasheed et al., 2013). The 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).

2.4. Conjugation experiments and plasmid typing

Conjugative plasmid transfer experiments were performed on Mueller-Hinton agar using the rifampicin resistant *E. coli* K12:W_{3110}*lac*$^{-}$*Rif*^{r} as recipient strain. Transconjugants were selected on MacConkey agar containing 2 mg/L cefotaxime or 0.5 mg/L imipenem and 50 mg/L rifampicin. Plasmid incompatibility groups were determined using the PCR-based replicon-typing scheme described by Carattoli et al. (2005). Detection of the R-replicon-type was done according to Garcia-Fernandes et al. (2009).

For plasmid fingerprinting, plasmid DNA of transconjugants was extracted with the Plasmid MidiPrep Kit (Quantum Prep BioRad Laboratories, Hercules, Ph, USA) and subjected to plasmid fingerprint analysis by restriction with *Pst*I (New England Biolabs, UK).

2.5. Molecular typing of the bacterial isolates

Total bacterial DNA was prepared using the Illustra Bacteria Genomic DNA Prep Kit (GE Healthcare, Little Chalfont, United Kingdom). ERIC PCR was performed as previously described (Markovska et al., 2015). The genetic similarity was determined using

Table 1
Distribution of carbapenemase–producing *K. pneumoniae* and *E. coli* isolates (one isolate per patient) by centre and year of isolation.

Centre	City	No. of beds	No. of isolates					
			Year of isolation					Total
			2014	2015	2016	2017	2018	
Sof-1	Medical institute, Ministry of the interior, Sofia	310	1	5	4	7	nd	17
Sof-2	Multiprofile Active Treatment Second City hospital, Sofia	280	-	-	-	1	nd	1
Sof-3	University Multiprofile Hospital for active treatment (UMHAT) “Ivan Rilski”, Sofia	395	-	1	1	9	3	14
Sof-4	University Multiprofile Hospital for active treatment and Emergency Medicine “Pirogov”, Sofia	795	nd	nd	nd	12	nd	12
VR	UMHAT “Saint Marina”, Varna	1250	18[a]	9	6	1	nd	16
PD	UMHAT “Saint George”, Plovdiv	820	-	20	11	5[b]	nd	36
PL	UMHAT “Georgi Stranski”, Pleven	1000	1[a]	3	-	6[c]	nd	9
StZ	UMHAT “Prof. d-r St Kirkovich”, Stara Zagora	280	-	-	-	-	-	-
Total			1	38	22	41	3	105

Abbreviations: nd: no data available; -: no carbapenem–non-susceptible *K. pneumoniae* or *E. coli* detected.
Sof: Sofia; VR: Varna; PD: Plovdiv; PL: Pleven; StZ: Stara Zagora.

[a] Carbapenemase-producing *K pneumoniae* and *E.coli* isolates from these centers have been previously published (Markovska et al., 2015; Markovska et al., 2017a,b) and have not been included in this survey.

[b] These isolates were collected until June 2017.

[c] Including one *E. coli* isolate.

Table 2
Antimicrobial susceptibility rates for 105 carbapenemase-producing isolates.

Antimicrobials	Resistant (no.) %	Intermediate resistant (no.) %	Susceptible (no.) %
Amoxicillin/clavulanic acid	(105) 100%	0	0
Piperacillin/tazobactam	(105) 100%	0	0
Cefotaxime	(105) 100%	0	0
Ceftazidime	(105) 100%	0	0
Cefepime	(105) 100%	0	0
Cefoxitin	(103) 98%	0	(2) 2%
Imipenem	(85) 81%	(20) 19%	0
Meropenem	(82) 78%	(22) 21%	(1) 1%
Tobramycin	(97) 92%	(3) 3%	(5) 5%
Gentamicin	(44) 42%	(25) 24%	(36) 34%
Amikacin	(29) 28%	(37) 35%	(39) 37%
Ciprofloxacin	(89) 85%	(5) 5%	(11) 10%
Levofloxacin	(78) 74%	(13) 12%	(14) 14%
Trimethoprim/ sulfamethoxazole	(88) 84%	0	(17) 16%
Chloramphenicol	(63) 60%	–	(42) 40%
Tigecycline[a]	(11) 11%	(35) 33%	(59) 56%
Colistin[a]	(13) 12%	–	(92) 88%

[a] The interpretation is on the basis of MIC results.

Dice coefficient as similarity measure and the unweighted pair group method with arithmetic mean (UPGMA) (http://genomes.urv.cat/UPGMA/).

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). The clonal complex analysis was performed by eBURST (http://eburst.mlst.net/). A clonal complex was defined as a group of two or more independent isolates that shared six identical alleles.

For the *E. coli* isolate, the typing was performed according to the protocol described on *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html). The allele sequences and STs were identified at the website (https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef).

3. Results

3.1. Strain collection

In total, 110 consecutive, non-duplicate carbapenem-nonsusceptible *K. pneumoniae* and four carbapenem-non-susceptible *E. coli* were collected from patients from seven of the participating hospitals in four towns. Carbapenem-nonsusceptible *K. pneumoniae* or *E. coli* isolates were not found at the hospital in Stara Zagora (Fig. 1).

The *K. pneumoniae* isolates were obtained from urine ($n = 29$), blood or central venous catheter ($n = 25$), wounds ($n = 20$), tracheal secretions or bronchoalveolar lavage ($n = 28$), peritoneal puncture ($n = 1$), pleural aspirate (n = 1) and sputum ($n = 6$). The *E. coli* isolates were collected from wounds ($n = 4$). The patients were hospitalized in Intensive Care Units ($n = 57$), surgical wards ($n = 26$), haemodialysis ward (n = 1), urology and nephrology wards ($n = 15$), internal medicine wards ($n = 14$) and one outpatient.

3.2. Phenotypic confirmation of carbapenemase production and antimicrobial susceptibility testing

Carbapenemase-production was confirmed for 105 (104 *K. pneumoniae* and one *E. coli*) of the 114 collected isolates. MBL-production was found in 51 (50 *K. pneumoniae* and one *E. coli*) isolates through the inhibition of the carbapenem-hydrolysing activity by dipicolinic acid. The carbapenemase activity of 53 isolates was inhibited by boronic acid. No inhibition of the carbapenem-hydrolysing activity by EDTA, dipicolinic acid, or boronic acid was observed for one *K. pneumoniae* isolate. All these isolates were further analysed.

The antimicrobial susceptibilities are shown in Table 2. Eighty-three of the isolates showed nonsusceptibility to three or more groups of antimicrobials categorizing them as multidrug-resistant bacteria, while 22 isolates remained susceptible to one or two antibiotic groups only classifying them as extensively drug-resistant.

3.3. Molecular identification of beta-lactamases

PCR experiments showed positive results for *bla*$_{\text{KPC}}$ group, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$,*bla*$_{\text{VIM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA-III}}$ group, *bla*$_{\text{CTX-M-1}}$ group, and *bla*$_{\text{CMY}}$ group. Sequence analysis identified *bla*$_{\text{VIM-1}}$*bla*$_{\text{KPC-2}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{SHV-1}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{CTX-M-3}}$,*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-4}}$, and *bla*$_{\text{OXA-1}}$ genes. Fifty-three *K. pneumoniae* isolates (51%) produced KPC-2 carbapenemase (Table 3). The majority of them were isolated in centres in Plovdiv ($n = 34$) and Varna ($n = 11$) but they were also

Table 3
Distribution of MLST-Types of 53 KPC-2-producing *K. pneumoniae* by centre and year of isolation.

Centre	Additional ESBLs	Year of isolation		
		2015	2016	2017
		MLST-type (No. of isolates)		
Sof-1 (4)	CTX-M-15 (4)	ST15 (4)		
Sof-2 (0)				
Sof-3 (0)				
Sof-4 (1)	CTX-M-3 (1)			ST258 (1)
VR (11)	CTX-M-15 (8)	ST15 (2) ST35 (1) ST76 (3)	ST15 (2)	
	CTX-M-15 + CTX-M-3 (3)		ST15 (1) ST395 (1)	ST15 (1)
PD (34)	CTX-M-15 (27)	ST29 (2) ST336 (9) ST902 (5)	ST29 (7)	ST29 (4)
	SHV-12 (2)		ST29 (2)	
	No (5)	ST16 (2) ST29 (1) ST902 (1)	ST29 (1)	
PL (3)	CTX-M-15 (3)	ST37 (3)		
	Total (53)	33	14	6

Abbreviations: Sof: Sofia; VR: Varna; PD: Plovdiv; PL: Pleven.

detectable in Sofia and Pleven. The highest number of KPC-2 producers was identified in 2015 thereafter their numbers have decreased. KPC-2 was most ofen co-produced with the ESBL CTX-M-15 (Table 3). Additionally, the majority of our bla$_{KPC-2}$ carrying *K. pneumoniae* produced *bla*$_{OXA-1}$ (*n* = 46). The intrinsic, chromosomal SHV-gene of KPC-producing *K. pneumoniae* was sequenced for 13 selected isolates and identified as *bla*$_{SHV-1}$ (n = 3) or *bla*$_{SHV-28}$ (*n* = 10).

The NDM-1 producers (47%; 48 *K. pneumoniae* and one *E. coli* isolates) were found in all participating centres, mostly in three centres in Sofia at an increasing frequency (Table 4). The NDM-1 carbapenemases were frequently co-produced with CMY-4, AmpC-enzyme (90%) and the ESBL CTX-M-15 (98%). In contrast to *bla*$_{SHV-1}$ and *bla*$_{SHV-28}$ found in KPC-2 producers, the NDM-1 producing *K. pneumoniae,* selected for sequencing of the chromosomal SHV, harboured *bla*$_{SHV-11}$ (*n* = 8). The isolates were negative for OXA-III group enzymes.

VIM-1 and OXA-48 carbapenemases were identified in two and one *K. pneumoniae* isolates, respectively, isolated in Varna. In both VIM-1 isolates the *bla*$_{SHV-12}$ gene was detected. The single isolate produced OXA-48 in combination with CTX-M-15.

3.4. Conjugation experiments, plasmid replicon typing and fingerprinting

The transfer of beta-lactamase genes was successful in 37% of the isolates (39 from 105). Among them, in 22 isolates the carbapenemase gene was transferred (Table 5). KPC-2 producing transconjugants were obtained for 12 of 53 wild-type isolates and *bla*$_{NDM-1}$ was transferred for nine of 48 *K. pneumoniae*. The *E. coli* isolate did not produce a transconjugant. An OXA-48 producing transconjugant was obtained, in contrast, the transfer of *bla*$_{VIM-1}$ was not achieved. The characteristics of transconjugants are shown in Table 5.

Plasmid replicon typing showed the presence of FIIAs replicon-type in all KPC-2 producing isolates (data not shown) as well as in all transconjugants that carried *bla*$_{KPC-2}$ (Table 5). The *Pst*I-restriction of the plasmids, isolated from 10 representative KPC-2 positive transconjugants, produced identical profiles.

For all but two *K. pneumoniae* and *E. coli* isolates (data not shown) and in all transconjugants harbouring *bla*NDM-1, the presence of A/C replicon-types was detected (Table 5). IncR replicon was found in *bla*$_{OXA-48}$ positive isolate. IncL/M replicon-types were associated with *bla*$_{CTX-M-3}$ transconjugations (Table 5).

3.5. Molecular typing and characterisation of the bacterial isolates

MLST-typing assigned the *K. pneumoniae* isolates to 13 different MLST-types.

The predominant MLST-cluster found was ST11, consisting of 47 NDM-1 producing *K. pneumoniae* isolates, exhibiting three very similar ERIC-types with a coefficient of similarity of 0,9–0,96 suggesting the presence of one clone. Only one NDM-1 producing isolate showed a different MLST-type (ST307) and ERIC type (coefficient of similarity of 0,4). The NDM-1 *K. pneumoniae* ST11 clone was detected in all centres but mainly in the hospitals in Sofia (Table 4).

The KPC-2 producing *K. pneumoniae* isolates (*n* = 53) were classified into 10 MLST types with ST29 (*n* = 17), ST15 (*n* = 10), ST336 (*n* = 9), and ST902 (*n* = 6) being the dominant types (Table 3). The MLST-types corresponded to 10 different ERIC-profiles (coefficient of similarity between 0.4 and 0.75). Three of the four dominating MLST-types among KPC-2 producers appeared only in the centre in Plovdiv (Table 3). Interestingly, the identification of *K. pneumoniae* intrinsic SHV-type beta-lactamase reflected in parts the results of the MLST-

Table 4
Distribution of 48 NDM-1-producing *K. pneumoniae* by centre and year of isolation.

Centre	Additional ESBLs	Year of isolation				
		2014	2015	2016	2017	2018
		MLST-type				
(No. of isolates)						
Sof-1 (13)	CTX-M-15 + CMY-4 (12)	ST11 (1)	ST11 (1)	ST11 (3)	ST11 (7)	
	CTX-M-15 + CTX-M + 3 + CMY-4 (1)			ST11 (1)		
Sof-2 (1)	CTX-M-15 + CMY-4 (1)				ST11 (1)	
Sof-3 (14)	CTX-M-15 + CMY-4 (12)		ST11 (1)		ST11 (8)	ST11 (3)
	CTX-M-15 + CTX-M + 3 + CMY-4 (1)				ST11 (1)	
	CTX-M-15 (1)				ST11 (1)	
Sof-4 (11)	CTX-M-15 + CMY-4 (10)				ST11 (10)	
	CMY-4 (1)				ST11 (1)	
VR (2)	CTX-M-15 + CMY-4 (2)			ST11 (2)		
PD (2)	CTX-M-15 + CMY-4 (1)			ST11 (1)		
	CTX-M-15 (1)				ST307 (1)	
PL (5)	CTX-M-15 + CMY-4 (3)				ST11 (3)	
	CTX-M-15 (2)				ST11 (2)	
	Total (48)	1	2	7	35	3

Abbreviations: Sof: Sofia; VR: Varna; PD: Plovdiv; PL: Pleven.

Table 5
Characteristics of 105 carbapenemase-producing isolates and their transconjugants.

Carbapene-mase/ Species	Additional ESBLs or AmpC	MLST-type	Transconjugants		
			Resisto-type	Beta-lactamases	Plasmid replicon-Types
	(No. of isolates)				
KPC-2 K. pn. (53)	CTX-M-15 (42)	ST29 (13)	AUG CTX CAZ IMP Pip/Taz (3)	KPC-2 (3)	FIIAs
		ST15 (8)	AUG CTX CAZ IMP Pip/Taz (2)	KPC-2 (2)	FIIAs
		ST902 (5)	-		
		ST37 (3)	AUG CTX CAZ IMP Pip/Taz (1)	KPC-2 (1)	FIIAs
		ST35 (1)	-		
		ST76 (3)	AUG CTX CAZ IMP Pip/Taz (1)	KPC-2 (1)	FIIAs
		ST336 (9)	AUG CTX CAZ IMP Pip/Taz (2)	KPC-2 (2)	FIIAs
	CTX-M-15 + CTX-M-3 (3)	ST15 (2)	CTX TOB GEN AMI (1)	CTX-M-3 (1)	L/M
		ST395 (1)	-		
	CTX-M-3 (1)	ST258 (1)	CTX TOB GEN AMI (1)	CTX-M-3 (1)	L/M
	SHV-12 (2)	ST29 (2)	AUG CTX IMP Pip/Taz (1)	KPC-2 (1)	FIIAs
			AUG CAZ TOB CHL (1)	SHV-12 (1)	A/C
	No (5)	ST16 (2)	-		
		ST902 (1)	-		
		ST29 (2)	AUG CTX CAZ IMP Pip/Taz (2)	KPC-2 (2)	FIIAs
NDM-1 K. pn. (48)	CTX-M-15 + CMY-4 (41)	ST11 (41)	AUG CAZ CTX TET SXT CHL IMP Pip/Taz (3)	NDM-1 + CTX-M-15 + CMY-4 (3)	A/C
			AUG CAZ CTX TOB AMI TET SXT CHL IMP Pip/Taz (6)	NDM-1 + CTX-M-15 + CMY-4 (6)	A/C
			AUG CAZ CTX TET SXT CHL (10)	CTX-M-15 + CMY-4 (10)	A/C
			AUG CAZ CTX TET SXT CHL (2)	CMY-4 (2)	A/C
	CTX-M-15 + CTX-M-3 + CMY-4 (2)	ST11 (2)	CTX TOB GEN AMI (1)	CTX-M-3 (1)	L/M
			AUG CAZ GEN TET SXT CHL (1)	CTX-M-3 + CMY-4 (1)	A/C + L/M
	CTX-M-15 (4)	ST11 (3)	-	-	-
		ST307 (1)	-	-	-
	CMY-4 (1)	ST11 (1)	-	-	-
VIM-1 K. pn. (2)	SHV-12 (2)	ST147 (2)	-	-	-
OXA-48 K. pn. (1)	CTX-M-15 (1)	ST15 (1)	IMP MERO (1)	OXA-48 (1)	R
NDM-1 E. co. (1)	CTX-M-15 (1)	ST10 (1)[a]	-	-	-

Abbreviations: K. pn., *K. pneumoniae*; E. co., *E. coli*; -, unsuccessful conjugation experiment; AUG, amoxicillin/clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; TOB, tobramycin; GEN, gentamicin; AMI, amikacin; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol; TET, tetracycline; IMP, imipenem; MERO, meropenem; Pip/Taz, Piperacillin/Tazobactam.

[a] Warwick scheme.

typing as only the ST11 *K. pneumoniae* harboured *bla*$_{\text{SHV-11}}$, while ST15 *K. pneumoniae* carried *bla*$_{\text{SHV-28}}$.

The single NDM-1 producing *E. coli* belonged to the ST10 lineage (Warwick scheme).

E-burst analyses showed that ST16 and ST336 *K. pneumoniae* might be combined to one clonal complex CC16 (two allele difference). The MLST-type ST11 was a single locus variant of ST258.

4. Discussion

In Bulgaria, the number of CRE has increased during the last years, as documented for invasive isolates by the European Antimicrobial Resistance Surveillance Network (EARS-Net) with reported carbapenem-resistance rates from 0% in 2013 to 4.4% in 2016 (ECDC, 2016).

During our study, 114 carbapenem-non-susceptible *Enterobacteriaceae* isolates were collected and among them 104 *K. pneumoniae* and one *E. coli* were confirmed to produce carbapenemases. In four of the hospital centres (PD, Sof-1, Sof-2, and Sof-3) carbapenemase-producing *K. pneumoniae* were detected for the first time highlighting that their spread in Bulgarian hospitals continues and has affected geographically distant hospitals.

The majority of our isolates (102/105) produced either KPC-2 or NDM-1 carbapenemases.

KPC-2 was identified in 51% of the *K. pneumoniae* isolates, with a decreasing frequency during the study period and predominantly from centres located outside Sofia. KPC-2 producing *K. pneumoniae* belonged to 10 different MLST-types with ST29, ST902, ST336, and ST15 being the dominant types in 79%. All MLST-types remained restricted to one center, except ST15 which was found in two hospitals (VR and Sof-1). For the centre in Plovdiv, where most of the KPC-2 producers were isolated and three of the dominating MLST-types (ST29, ST902, ST336) were found, an endemic clonal spread or an on-going outbreaks might be supposed.

K. pneumoniae isolates of MLST-type ST15 are known to represent a very successful clone widely disseminated in Europe and associated with the production of different carbapenemases (KPC-2, OXA-48, VIM-1) (Esteban-Cantos et al., 2017; Melegh et al., 2014; Potron et al., 2013; Rodrigues et al., 2014; Wang et al., 2015). However, in our study, ST15 *K. pneumoniae* remained restricted to two hospitals (VR and Sof-1).

While KPC-2 producing ST258 *K. pneumoniae* have been found during several European studies (Baraniak et al., 2017; Logan and Weinstein, 2017; Kaase et al., 2016; Pitout et al., 2015), we detected a single ST258 isolate.

Our analyses proved the location of *bla*$_{\text{KPC-2}}$ on IncFII-plasmids with identical plasmid fingerprints. It seems that the spread of KPC-2 is more attributable to the dissemination of IncFII-plasmids than to the expansion of single clones. This highlights the role of IncFII-plasmids for the distribution of *bla*$_{\text{KPC-2}}$ and is in concordance with the results reported by other authors (Pitout et al., 2015; Baraniak et al., 2017).

Among our isolates the carbapenemase NDM-1 was found in 48 *K. pneumoniae* and one *E. coli*. All but one of our NDM-1 producing *K. pneumoniae* belonged to MLST-type 11. The ST11 *K. pneumoniae*, a one locus variant of ST258, is an example of a very successful epidemic clone. It has been reported that this clone carries different carbapenemase genes like bla_{KPC}, bla_{VIM}, $bla_{\mathrm{OXA\text{-}48}}$, and bla_{NDM} (Lu et al., 2018; Solgi et al., 2018; Wang et al., 2015).In Europe, outbreaks caused by NDM-1 and CTX-M-15 producing ST11 *K. pneumoniae* isolates have been reported from Greece (Voulgari et al., 2014) and Poland (Baraniak et al., 2016). Studentova et al. (2015) also identified two cases of NDM-1 producing ST11 *K. pneumoniae* in Czech Republic. In Bulgaria, the detection of NDM-1 producing ST11 *K. pneumoniae* was first described by Todorova et al. (2016), Kostyanev et al. (2016). Savov et al. (2018) reported a hospital outbreak in Bulgaria, caused by NDM-1 producing ST11 *K. pneumoniae* isolates with characteristics similar to the Greek clonal ST11 strain. Our study have revealed a multi-centre dissemination of clonally related NDM-1 producing ST11 *K. pneumoniae*, with the first isolate detected in centre Sof-1 in Sofia as early as in 2014. The isolation frequency has increased over the years, affecting seven medical centres in four towns in 2017, suggesting an interregional circulation of NDM-1 producing ST11 *K. pneumoniae* (according to the definition of Albiger et al. (2015). In addition to the carbapenemase NDM-1 and the ESBL CTX-M-15, our isolates co-produced the AmpC-type beta-lactamase CMY-4. This has been reported for the Czech isolates (Studentova et al., 2015) but not for the Polish and Greek isolates (Baraniak et al., 2016; Voulgari et al., 2014). We may suggest that the combination of three different enzymes (carbapenemase, AmpC, and ESBL) in the same isolates, contribute to their dissemination.

Clonal *K. pneumoniae* spread seemed to be the major mechanism for NDM-1 dissemination in the participating hospitals. Nevertheless, our conjugation experiments showed the location of $bla_{\mathrm{NDM\text{-}1}}$, $bla_{\mathrm{CTX\text{-}M\text{-}15}}$ and $bla_{\mathrm{CMY\text{-}4}}$ on transferable plasmids of the A/C replicon-type. Dissemination of $bla_{\mathrm{NDM\text{-}1}}$ gene by IncA/C plasmids has been previously reported by several authors (Mulvey et al., 2011; Giske et al., 2012; Todorova et al., 2016).

During our study, a single NDM-1 producing *E. coli* isolate of MLST-type 10 was found in the center in Pleven. Similarly, in Poland, NDM-1 producing ST10 *E. coli* have already been detected (Baraniak et al., 2016). In contrast, the previously detected *E. coli* in Sofia (Poirel et al., 2014) belonged to MLST-type 101.

During the entire study period, we identified only three isolates producing carbapenemases other than NDM-1 and KPC-2 in the center in Varna. Two of them were VIM-1 producing ST147 *K. pneumoniae* isolates. In the same hospital, VIM-1 producing *K. pneumoniae* have already been identified in 2012 (Markovska et al., 2015). Similar strains have been reported by Papagiannitsis et al. (2013) in Greece. The third *K. pneumoniae* isolate produced OXA-48 and belonged to MLST-type ST15. In contrast to our results, OXA-48 producers have been widely disseminated in some European countries (Poland, Germany), in Turkey (Ece et al., 2018; Izdebski et al., 2017; Kaase et al., 2016; Potron et al., 2013).

We should also emphasize that the antibiotic selective pressure in the Bulgarian hospitals may contribute to the further expansion of specific clones., The use of the third generation cephalosporins is one of the highest among European countries (https://ecdc.europa.eu/en/publications-data/summary-latest-data-antibiotic-consumption-eu-2017).

An alarming finding is that 83 of our isolates were multidrug-resistant and 22 extensively drug resistant. Even colistin, one of the last-line option for treatment of CRE infected patients (depending on the site of infection and the kidney function) was found to be ineffective against 13 of our isolates.

In conclusion, the clonal interregional expansion of NDM-1 producing ST11 *K. pneumoniae* and the dissemination of $bla_{\mathrm{KPC\text{-}2}}$ carrying plasmids seem to be responsible for the spread of carbapenemase-producing *K. pneumoniae* in Bulgaria. Our data highlight the urgency for measures to prevent their further dissemination.

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Conflict of interest

None declared.

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