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Article

An Intra-Hospital Spread of Colistin-Resistant *K. pneumoniae* Isolates—Epidemiological, Clinical, and Genetic Analysis

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Abstract: *Background and objective:* K. pneumoniae appeared to be a significant problem due to its ability to accumulate antibiotic-resistance genes. After 2013, alarming colistin resistance rates among carbapenem-resistant K. pneumoniae have been reported in the Balkans. The study aims to perform an epidemiological, clinical, and genetic analysis of a local outbreak of COLr CR-Kp. *Material and methods:* All carbapenem-resistant and colistin-resistant K. pneumoniae isolates observed among patients in the ICU unit of Military Medical Academy, Sofia from 1 January to 31 October 2023 were included. The results were analyzed according to the EUCAST criteria. All isolates were screened for blaVIM, blaIMP, blaKPC, blaNDM, and blaOXA-48. Genetic similarity was determined using the Dice coefficient as a similarity measure and the unweighted pair group method with arithmetic mean (UPGMA). mgrB genes and plasmid-mediated colistin resistance determinants (mcr-1, mcr-2, mcr-3, mcr-4, mcr-5) were investigated. *Results:* There was a total of 379 MDR-K. pneumoniae isolates, 88% of which were carbapenem-resistant. Of them, there were 9 (2.7%) colistin-resistant isolates in six patients. A time and space cluster for five patients was found. Epidemiology typing showed that two isolates belonged to clone A (pts. 1, 5) and the rest to clone B (pts. 2-4) with 69% similarity. Clone A were coproducers of blaNDM-like and blaOXA-48-like and had mgrB-mediated colistin resistance (40%). Clone B isolates had only blaOXA-48-like and intact mgrB genes. All isolates were negative for mcr-1,-2,-3,-4,-5 genes. *Conclusions:* The study describes a within-hospital spread of two clones of COLr CR-Kp with a 60% mortality rate. Clone A were coproducers of NDM-1 and OXA-48-like enzymes and had mgrB-mediated colistin resistance. Clone B isolates had only OXA-48-like enzymes and intact mgrB genes. No plasmid-mediated was found. The extremely high mortality rate and limited treatment options warrant strict measures to prevent outbreaks.

Keywords: outbreak; colistin-resistant K. pneumoniae; mgrB,plasmid-mediated resistance; mortality

1. Introduction

HAIs (Hospital-acquired infections) account for 5-15% of all admissions worldwide (9 million), but the rate is probably higher because of significant underreporting [1]. The total annual cost for the

five significant HAIs in the USA is $9.8 billion [2]. Almost 100 years after the discovery of antibiotics we are faced with unprecedented antibiotic resistance led to a catastrophic crisis worldwide. Multidrug resistance (MDR) is defined as resistance to one or more antimicrobials from at least three different antimicrobial classes, extensive drug resistance (XDR) is non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories), while pan drug resistance (PDR) to all agents in all antimicrobial categories (PDR) [3]. MDR pathogens account for 670,000 infections and 33,000 deaths in the European Union with healthcare costs of $ 1.1 billion [4,5]. In 2022, Antimicrobial Resistance Collaborators estimated that 1.27 million deaths were attributable to antibiotic resistance during 2019. Six of 23 analyzed pathogens (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*) were responsible for 929,000 of these deaths [6].

In 2016, a UK report warned that by 2050 approximately 10 million deaths would occur if no action was taken [7]. These figures, however, were questioned by de Kraker et al. mainly due to a lack of reliable estimates of the antibiotic resistance burden [8]. Nevertheless, WHO declared priority status to the most frequently reported MDR bacteria called "ESKAPE" (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*) due to their ability to escape the antibiotics [9,10].

The MDR Enterobacteriaceae are essential because they are part of the human microbiota. Among them, *K. pneumoniae* appeared to be a significant problem due to its ability to accumulate antibiotic resistance genes (ARGs) by de novo mutations or via horizontal gene transfer by plasmid transfer (conjugation) or by bacteriophage (transduction), so Navon-Venezia et al. called it a *"source and shuttle for antibiotic resistance"* [11]. Actually, via the above-mentioned mechanisms, ARGs could be easily transferred from harmless commensals to pathogenic bacteria [12].

According to a recent survey published in the Lancet, *K. pneumoniae* is among the five deadliest bacteria with more than 500,000 deaths yearly [13]. The carbapenem-resistant *K. pneumoniae* was first reported in 2001 in the USA [14]. The European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE) demonstrated that the epidemic of carbapenem-resistant *Klebsiella pneumoniae* in Europe is driven by *"within-hospital transmission and interhospital spread rather than between countries"* [15]. According to ECDC (2021), the MDR strains are 21% but there is an increasing trend for carbapenemase-producing strains (11.7%), varying between 0% and 74% [5]. Carbapenem-resistant *K. pneumoniae* causes 50,000-100,000 deaths worldwide annually [6].

The Balkans are considered to be a reservoir of MDR and XDR *K. pneumoniae*. NDM-1-producing strains appear to spread within a similar time frame (2013-2016) [4,16–18]. The first polyclonal outbreak caused by NDM-1-producing *K. pneumoniae* in Bulgaria was described in 2015 at our and other Institutions [16,17]. In 2019, Markovska et al. demonstrated the rapid interregional spread of NDM-1-producing ST11 strains with plasmid-mediated carbapenem resistance [19]. Unfortunately, the last link of the chain was the emergence of Colistin-resistant strains. Polymyxin was discovered in 1947 and has been available on the market since 1959 for the treatment of Gram-negative infections [20]. Due to its nephrotoxicity, in the 1970s it was replaced by other antibiotics. Due to the emerging MDR crisis, since 2000 Colistin was "re-discovered" and began to play a strategic role in the treatment of MDR Gram-negative infections [21,22]. At the same time, the rapid increase in its use rapidly led to an increase in resistance [20]. The studies published in the period 2008-2011 demonstrated colistin-resistant *K. pneumoniae* (COLr CR-Kp) between 1.5% and 28% [20]. According to Binsker et al. citing the ATLAS database, the global colistin resistance rate for 2014-2019 varies between 2.6% and 4.6%, for Europe (2.4-3.4%) [23]. The first two cases in Bulgaria were reported from our Institution in 2016 followed by other hospitals in Sofia [24–26]. After 2013, alarming colistin resistance among carbapenem-resistant *K. pneumoniae* has been reported in the Balkans – Bulgaria (37%), Greece (40%), Romania (27.5%), Serbia (10.6%), Türkiye (25.5%) and Italy (27%) [23,26–30]. The imminent disaster tolls for emergent measures to prevent its spread and to find new treatments because of the high mortality rate (41-70%) [31,32].

This study aims to perform an epidemiological, clinical, and genetic analysis of a local outbreak of COLr CR-Kp.

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2. Material and methods

2.1. Bacterial isolates and patients

The colistin-resistant and carbapenem-resistant *K. pneumoniae* isolates observed among patients in the ICU unit of Military Medical Academy, Sofia from 1 January to 31 October 2023 were included in the study.

The identifications of the microbial isolates were performed by MALDI-TOF mass spectrometry (MALDI-TOF MS, Bruker), following the manufacturer's instructions. Antimicrobial susceptibility testing

The Antimicrobial susceptibility testing (AST) was determined by Vitek 2 (bioMerieux). AST of Colistin was performed by the reference method broth microdilution (ComASP Colistin, Liofilchem). The results were analyzed according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [33].

2.2. Molecular-genetic investigations

All isolates were PCR screened for the presence of *bla*VIM, *bla*IMP, *bla*KPC, blaNDM and blaOXA-48, as previously described [34]. The MgrB gene was amplified and sequenced with primers reported previously by Kanateli. Nucleotide and deduced amino acid sequences were analyzed 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).

Total bacterial DNA was prepared using the boiling method. ERIC PCR with ERIC1R and ERIC2 primer set was performed as previously described [19]. Genetic similarity was determined using the Dice coefficient as a 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.

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. [35]. Chromosomal *mgrB* genes were amplified with primer sets as described previously [36].

3. Results

3.1. Bacterial isolates and patient's characteristics

A total of 379 MDR-*K. pneumoniae* isolates were isolated in the ICU unit, of which 333 (87.9%) were carbapenem-resistant. Of them, there were 9 (2.7%) colistin-resistant isolates in six patients. A time and space cluster for five patients was observed. They were treated in the same room in the ICU within a two-week overlapping interval. All COLr CR-Kp were isolated within 13 days (31.08-13.09). The characteristics of the patients are shown in Table 1**.** All patients, except the fifth, had previous endoscopic interventions and initially colistin-susceptible *K. pneumoniae,* but on the table are given only COLr CR-Kp.

Table 1.

n.	g	age	diagnose	intervention	previous intervention	Microbiology: sample	Microbiology: date	ICU stay	outcome	treatment	carbapenemase	ERIC**	Mcr 1-5 plasmid	mgrB
1	f	69	Acute kidney bleeding	Left nephrectomy	*RIRS + LT + JJ cystoscopy	wound blood culture	31.08 8.09	18.08-3.10	died	unazyn, ceftriaxon, meropenem, colistin	OXA-48-like NDM-1	A	NEG	NEG

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3	m	71	Pancreatic cancer	Traverso-Longmire	bile duct stent	wound tracheo-bronchial tree	3.09 12.09-2.10	1-5.09 12.09-2.10	died	piperacilin/tazobactam, sulcef, linezolid, colistin	OXA-48-like	B	NEG	POS
2	m	46	Pancreatic cancer	Traverso-Longmire	bile duct stent	wound	12.09	18-19.08 12-14.09	discharged	piperacilin/tazobactam, ciprofloxacin, colistin	OXA-48-like	B	NEG	POS
4	m	82	Urine bladder cancer	Cystectomy	-	tracheo-bronchial tree blood culture	4.09 13.09	28.08-14.09	died	unazyn, levofloxacin, doxycycline, colisitn	OXA-48-like	B	NEG	POS
5	m	56	Perforated duodenal ulcer	Suture	-	urine	11.09	5-15.09	discharged	meropenem	OXA-48-like NDM-1	A	NEG	NEG

The supposed index patient was a 69-year-old woman, who underwent a left nephrectomy due to acute bleeding after retrograde intrarenal surgery, lithotripsy, and JJ stent. The urine culture revealed colistin-susceptible, carbapenem-resistant *K. pneumoniae* (22.08, data not shown), which was treated with a suboptimal dose regimen of Colistin (2x1 MM U) due to kidney failure. The first COLr CR-Kp was isolated from the wound tissue (31.08). The wound was treated locally but after seven days a reoperation was performed due to organ space infection and severe necrotizing fasciitis. The patient developed sepsis with a positive blood culture (8.09) caused by the same strain and died on the 42nd POD.

The second and third patients underwent a Traverso-Longmire procedure for pancreatic cancer, both had bile duct stenting one month before the operation. The first isolates from the bile during the index operation in both patients were Colistin-susceptible, carbapenem-resistant *K. pneumoniae* (data not shown). In the second patient, COLr CR-Kp was recovered from the wound (3.09). In the third patient, it was also isolated from the wound (12.09). Both patients were treated in one surgical clinic and both underwent reoperation on the same day (12.09).

In the fourth patient, colistin-susceptible, carbapenem-resistant *K. pneumoniae* was found in the urine (31.09), but COLr CR-Kp was isolated from the tracheobronchial tree (4.09), on the 7th POD followed by positive blood culture (13.09). In the fifth patient, COLr CR-Kp was isolated from the urine on the 6th POD (11.09). Three of the five patients died (60%).

3.2. Antimicrobial susceptibility testing

All COLr CR-Kp isolates were PDR as follows: Amikacin: [R], Amoxicillin: [R], Ampicillin: [R], Ampicillin/Sulbactam: [R], Amoxicillin/Clavulanic acid (I.V.): [R], Amoxicillin/Clavulanic acid (oral): [R], Cefepime: [R], Cefixime: [R], Cefoxitin: [R], Ceftazidime: [R], Ceftriaxone: [R], Cefuroxime (oral): [R], Cefuroxime (I.V.): [R], Cefoperazon/Sulbactam: [R], Cefpodoxime: [R], Cefazolin: [R], Colistin: [R], Gentamicin: [R], Imipenem: [R], Levofloxacin: [R], Meropenem: [R], Etrapenem: [R], Fosfomycin (oral): [R], Ciprofloxacin: [R], Moxifloxacin: [R], Norfloxacin: [R], Nalidix acid: [R], Piperacillin/Tazobactam: [R], Tetracycline: [R], Doxycycline: [R], Trimethoprim/Sulfamethoxazole: [R].

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3.3. Molecular-genetic investigations

PCR reactions confirmed the production of carbapenemases in all five isolates. Clone A was a coproducer of *bla*$_{NDM\text{-}like}$ and *bla*$_{OXA\text{-}48\text{-}like}$ enzymes (pts. 1 and 5), whereas clone B (pts. 2-4) harbored only *bla*$_{OXA\text{-}48\text{-}like}$ enzymes. Upgma analysis showed a 0.69 similarity coefficient between the two clones. The entire *mgrB* gene was amplified by PCR. In the isolates from clone A the *mgrB* genes could not be amplified, showing truncated genes. In clone B there were amplicons in all three isolates (intact *mgrB* genes), (Table 1). All isolates were negative for mcr-1,-2,-3,-4,-5 genes.

A broad hospital infection-control campaign was initiated encompassing the other patients, staff, and working environment but no other COLr CR-Kp strains were isolated.

4. Discussion

The present analysis revealed two clones of COLr CR-Kp that coincide with the time and space in the ICU unit. The most important was clone A, co-producer of OXA-48 and NDM-1, and harbored disrupted *mgrB* genes responsible for the colistin resistance. The isolates of this clone were resistant to all tested antimicrobials leaving no therapeutic alternative. Clone B harbored only OXA-48 and lacked mutations in *mgrB* genes, so the colistin resistance might be explained by other chromosomal mutations [35,36]. The finding suggests that our series represents a within-hospital spread of COLr CR-Kp with two foci that coincide in the time and space (ICU). We speculate that the second focus originates from the second and third patients, who were operated on and managed in one clinic by the same team with subsequent stays in the ICU close to the fourth patient.

Of note, all patients in the presented series, except the fifth, underwent endoscopic intervention before the index operation (two endourology procedures and two bile duct stenting). All of them had an initial culture of colistin-susceptible *K. pneumoniae*, which also demonstrates the within-hospital spread.

Our finding is similar to the EuSCAPE survey, which demonstrated the central role of the inter-hospital but more pronounced transmission at the hospital level, similar to Markovska et al. [15,19]. Other authors, however, reported that *"carbapenem resistance reveals remarkable diversity and unexplained mechanisms"* and not all outbreaks could be linked to transmissions [37].

The first and fourth patients were treated with suboptimal doses of colistin and we can speculate that the transition from colistin-susceptible to COLr CR-Kp in our series might be explained by this selective antibiotic pressure [38].

Although it was declared as strategic by WHO, colistin has been increasingly used in clinical practice, with a steep increase after 2005 [19]. Moreover, in 2017, the overall consumption of polymyxins in food-producing animals in 28 EU countries was 340 times higher than in human medicine [24]. A logical consequence of this worrisome trend is the rapid emergence of Colistin-resistant strains. A recent meta-analysis demonstrated a significant increase in bloodstream COLr CR-Kp during the last decade from 3% in 2015 to 13% in 2020 and after [39]. In the Balkans, the first COLr CR-Kp strains were isolated in 2012 with rapid expansion leading to multiple outbreaks in Greece, Bulgaria, and probably in other countries (Table 2), [26–30,40].

Table 2. The rate of Colistin resistance in carbapenem-resistant *K. pneumoniae* in Balkan countries.

author	country, year	% of MDR
Markovska, et al. [26]	Bulgaria, 2022	37
Galani, et al. [28]	Greece, 2014-2016	40.4
epi-net.eu/records/12313/12313/ [27]	Romania, 2018	27.5
Palmieri, et al. [30]	Serbia, 2013-2017	10.6
Cizmeci, et al. [29]	Türkiye, 2016	27.5

In a recent meta-analysis, Yusof et al. reported a pooled prevalence of mutated colistin resistance in *K. pneumoniae* of about 75% [41]. The most common genetic mechanism of resistance includes

mutations in the genes *mgrB* (88%), *pmrA*/*pmrB* (54%), *phoQ* (44%), and *phoP* (36%). Plasmid-mediated resistance via *mcr-1* was noted in 14%, while other genetic mechanisms in 40%. In Bulgaria, a recent study by Markovska et al. found a lack, disrupted, or mutated of the *mgrB* gene in 9/37 cases (24%), whereas in the rest the mechanism of resistance was not elucidated [26]. No plasmid-mediated resistance was found. This data is similar to our results (*mgrB* 40%).

The mortality rate of the present series is 60% and 100% in cases with bloodstream infection, which is in unison with the literature [31,32,42]. As of today, a few treatment options exist such as Ceftazidime/avibactam with or without Aztreonam, Plazomicin (not approved by EMA), Cefideracol, and Fosfomycin. If MIC of Imipenem is below 8 mg/L it could also be included in combination schemes. An excellent review of Petrosillo et al. demonstrated their characteristics and highlighted the need for an analysis of OXA-48 like and NDM status to guide the treatment [43].

The extremely high mortality rate and limited treatment options warrant strict measures to prevent outbreaks. Given the overtaking bacterial resistance and the difficult control of the chaotic and frequently defensive use of antibiotics, it appears more prudent to improve the prevention. Despite the high risk of bias (99%), the published literature suggests a sustained potential for reduction of HAI rates between 35% and 55% by multifaceted interventions irrespective of a country's income level [44].

5. Conclusions

The present study describes a within-hospital spread of two clones of COLr CR-Kp with a 60% mortality rate. Clone A were co-producers of NDM-1 and OXA-48-like enzymes and had mgrB-mediated colistin resistance. Clone B isolates had only OXA-48-like enzymes and intact mgrB genes. No plasmid-mediated resistance was found. The study also confirms the central role of the transmission at the hospital level, not only for COLr CR-Kp but also for colistin-susceptible *K. pneumoniae*. The extremely high mortality rate and limited treatment options warrant strict measures to prevent outbreaks.

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