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Molecular epidemiology of carbapenem-resistant *Enterobacteriaceae* isolated from patients in COVID-19 wards and ICUs in a Bulgarian University Hospital

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RESEARCH ARTICLE



ABSTRACT

Many studies report an increase in antimicrobial resistance of Gram – negative bacteria during the COVID-19 pandemic. Our aim was to evaluate the epidemiological relationship between carbapenem-resistant (CR) *Enterobacteriaceae* isolates from patients in COVID-19 wards and to investigate the main mechanisms of carbapenem resistance in these isolates during the period April 2020–July 2021. A total of 45 isolates were studied: *Klebsiella pneumoniae* ($n = 37$), *Klebsiella oxytoca* ($n = 2$), *Enterobacter cloacae* complex ($n = 4$) and *Escherichia coli* ($n = 2$). Multiplex PCR was used for detection of genes encoding carbapenemases from different classes (bla_{KPC} , bla_{IMP} , bla_{VIM} , bla_{NDM} , bla_{OXA-48}). For epidemiological typing and analysis, ERIC PCR was performed. Two clinical isolates of *E. cloacae*, previously identified as representatives of two dominant hospital clones from the period 2014–2017, were included in the study for comparison. In the CR *K. pneumoniae* group, 23 (62.2%) carried bla_{KPC} , 13 (35.1%) bla_{NDM} , 10 (27.0%) bla_{VIM} , and 9 (24.3%) were positive for both bla_{KPC} and bla_{VIM} . The bla_{KPC} was identified also in the two isolates of *K. oxytoca* and bla_{VIM} in all *E. cloacae* complex isolates. The two CR isolates of *E. coli* possessed bla_{KPC} and bla_{OXA-48} genes. Epidemiological typing identified 18 ERIC profiles among *K. pneumoniae*, some presented as clusters of identical and/or closely related isolates. The carbapenem resistance in the studied collection of isolates is mediated mainly by bla_{KPC} . During the COVID-19 pandemic intrahospital dissemination of CR *K. pneumoniae*, producing carbapenemases of different molecular classes, as well as continuing circulation of dominant hospital clones of multidrug-resistant *E. cloacae* complex was documented.

KEYWORDS

COVID-19 wards, carbapenem-resistant *Enterobacteriaceae*, intrahospital dissemination

INTRODUCTION

Since its emergence in 2020, the SARS-CoV-2 pandemic had a significant impact on each aspect of the public healthcare worldwide, raising long-term issues such as a constantly increasing antimicrobial resistance [1]. Patients with severe COVID-19 in critical conditions usually need prolonged hospitalization, which is often complicated by nosocomial bacterial infections. Different authors report that more than 70% of patients admitted to hospitals have been treated with antibacterial agents, with 33% self-prescribing therapy before hospital admission [2]. The overall rate of bacterial infections among COVID-19 patients was estimated to be 6.9%, varying by patient population, ranging from 5.9% in hospitalized patients to 8.1% in critically ill patients [3]. Some authors report even higher incidence of bacterial infections in intensive care unit (ICU) patients (13.5%–44%), mainly associated with multidrug-resistant (MDR) Gram – negative bacteria [4, 5]. These patients were associated

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with increased risk for severe infections, caused by carbapenemase-producing *Enterobacteriaceae* and with high mortality rate [6]. Many studies documented an increase in antimicrobial resistance during the COVID-19 pandemic in Gram – negative bacteria, a phenomenon associated with the excessive and unnecessary antimicrobial therapy, non-compliance with hygiene measures, prolonged stay in ICUs, decreased resistance surveillance due to diagnostic focus on COVID-19 and overloading of healthcare systems [7].

The infections caused by MDR carbapenemase-producing bacteria have limited treatment options [8]. This problem existed before the onset of SARS-CoV-2. In some countries (Israel, Greece, Italy, Turkey) the incidence of carbapenem-resistant (CR) *Klebsiella pneumoniae* exceeded 50% before the pandemic [9]. The European Centre for Disease Prevention and Control reported a statistically significant trend for increasing the rate of CR *K. pneumoniae* invasive isolates in Europe during the period 2015–2020 (from 6.8% in 2015 to 10% in 2020), with the highest rates detected in Greece (66.3%), Romania (48.3%) and Italy (29.5%) [10]. Before the pandemic, Bulgaria also reported CR *K. pneumoniae* isolates, associated with blood stream infections. During the same period, the rate of CR blood isolates of *Klebsiella pneumoniae* significantly increased from 3.2% in 2015 to 28.1% in 2020 [10–12].

In 2020 the World Health Organization announced the threat of antimicrobial resistance as one of the most pressing challenges of our time, further exacerbated by the COVID-19 pandemic [13]. This fact determines the surveillance of antimicrobial resistance and the investigation of its mechanisms as priorities for both individual patient care and infection control strategies on global, national and regional levels.

The aim of this study was to evaluate the epidemiological relationship between carbapenem-resistant *Enterobacteriaceae* isolates, obtained from patients hospitalized in COVID-19 wards and ICUs in Varna University Hospital, Bulgaria during the period April 2020–July 2021 and to investigate the main carbapenem resistance mechanisms in these isolates.

MATERIALS AND METHODS

A total of 143 non-duplicate CR *Enterobacteriaceae* isolates were obtained from hospitalized patients in the ICUs and the COVID-19 wards in the hospital during the studied period: *K. pneumoniae* ($n = 135$), *Klebsiella oxytoca* ($n = 2$), *Enterobacter cloacae* complex ($n = 4$), *Escherichia coli* ($n = 2$). Based on the following criteria - resistance profile, type of the ward and specimen, year of isolation, a total of 45 CR isolates were studied: *K. pneumoniae* ($n = 37$), *K. oxytoca* ($n = 2$), *E. cloacae* complex ($n = 4$) and *E. coli* ($n = 2$). The isolates were obtained from urine samples ($n = 23$), blood ($n = 4$), respiratory samples ($n = 6$), wound secretions ($n = 6$), punctates ($n = 3$), feces ($n = 1$), semen ($n = 1$) and from the hospital environment ($n = 1$). Two clinical isolates of *E. cloacae*, previously identified as representatives of two dominant hospital clones from the

period 2014–2017, were included in the study for comparison. Species identification and antimicrobial susceptibility testing were done by Phoenix automated system (Beckton Dickinson, USA) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. Confirmation of species identification was performed by MALDI Biotyper Sirius (Bruker, Germany). The susceptibility to colistin was determined by the broth microdilution method (Erba Lachema, Czech Republic). Multiplex PCR was performed to detect the presence of genes encoding carbapenemases from different classes (*bla*_{KPC}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{OXA-48}) [14]. Epidemiological typing was done by ERIC PCR [15]. The ERIC patterns of the studied isolates were subjected to UPGMA analysis. Similarity of >70% was used as a threshold for clonal relatedness of the isolates. The study was approved by the Ethical Committee of Varna Medical University (124/06.01.2023).

RESULTS

A total of 45 isolates (*K. pneumoniae*, $n = 37$, *K. oxytoca*, $n = 2$, *E. cloacae* complex, $n = 4$, *E. coli*, $n = 2$) were studied for their main CR mechanisms and genetic relatedness.

The rates of antimicrobial resistance in the studied collection of CR isolates, presented in increasing order, were as follows: colistin, 7%; amikacin, 52%; trimethoprim/sulphamethoxazole, 56%; ceftazidime/avibactam, 60%; gentamicin, 69%; tobramycin, 98%; fluoroquinolones (ciprofloxacin, levofloxacin), 100%.

In the CR *K. pneumoniae* group, 23 isolates (62.2%) carried *bla*_{KPC}, 13 (35.1%) - *bla*_{NDM}, 10 (27.0%) - *bla*_{VIM}, and 9 isolates (24.3%) were positive for both *bla*_{KPC} and *bla*_{VIM}. The *bla*_{KPC} was identified also in the two isolates of *K. oxytoca* and *bla*_{VIM} in all *E. cloacae* complex isolates. The two CR *E. coli* isolates possessed *bla*_{KPC} and *bla*_{OXA-48} genes (Table 1).

Eighteen different ERIC types (A–R) were detected among the isolates of *K. pneumoniae*, some presented as clusters of identical and/or closely related isolates (A, B, C, D, E, F) (Table 1). Twelve isolates demonstrated unique ERIC profiles. Type C was the predominant, found in 8 isolates, collected from patients, hospitalized in 5 hospital wards. ERIC type D was identified in 6 isolates from 4 hospital wards. *K. pneumoniae*, isolated from the hospital environment in the ICU in March 2021 also demonstrated ERIC type D. Similarly, ERIC types A and B were detected in different hospital wards. ERIC types A and E were identified in 2020, while D and F – in 2021. In contrast, isolates with ERIC profiles B and C were detected during the whole studied period (Table 1).

All four *E. cloacae* complex isolates were demonstrated with different ERIC profiles (Table 1). Two of these isolates exhibited identical profiles with those of previously isolated *E. cloacae*, representatives of two dominant hospital clones from the period 2014–2017. One ERIC profile was found in *K. oxytoca* and *E. coli* (Table 1).



Table 1. Characteristics of *Enterobacteriaceae* isolates analyzed in this study

Bacterial species	ERIC type (n)	<i>bla</i> genes (n)	Year of isolation (n)	Department (n)
<i>K. pneumoniae</i>	A (n = 2)	<i>bla</i> _{NDM} (n = 2)	2020 _n = 2	Neurology _n = 1; INU _n = 1
	B (n = 4)	<i>bla</i> _{KPC} (n = 4)	2020 _n = 2; 2021 _n = 2	Pediatric ward _n = 1; Pediatric Hematology _n = 1; IPU _n = 2
	C (n = 8)	<i>bla</i> _{KPC} + <i>bla</i> _{VIM} (n = 8)	2020 _n = 4; 2021 _n = 4	Hemodialysis _n = 1; Nephrology _n = 3; ICU _n = 2; Surgery _n = 1; Cardiology _n = 1
	D (n = 6*)	<i>bla</i> _{NDM} (n = 6)	2021 _n = 6	INU _n = 2; Hematology _n = 1; ICU _n = 2; Nephrology _n = 1
	E (n = 2)	<i>bla</i> _{NDM} (n = 2)	2020 _n = 2	INU _n = 2
	F (n = 3)	<i>bla</i> _{KPC} (n = 3)	2021 _n = 3	IPU _n = 3
	unique profiles (n = 12)	<i>bla</i> _{KPC} (n = 7); <i>bla</i> _{NDM} (n = 3); <i>bla</i> _{VIM} (n = 1) <i>bla</i> _{KPC} + <i>bla</i> _{VIM} (n = 1)	2020 _n = 10; 2021 _n = 2	Hematology _n = 1; Therapeutic ward _n = 2; ICU _n = 1; Hemodialysis _n = 1; Pediatric ward _n = 1; Urology _n = 1; INU _n = 2; Neurology _n = 1; Gastroenterology _n = 1; Rheumatology _n = 1
<i>K. oxytoca</i>	A (n = 2)	<i>bla</i> _{KPC} (n = 2)	2021 _n = 2	IPU _n = 2
<i>E. cloacae</i> complex	a** (n = 1)	<i>bla</i> _{VIM} (n = 1)	2020 _n = 1	Transplantology ward _n = 1
	b (n = 1)	<i>bla</i> _{VIM} (n = 1)	2021 _n = 1	Therapeutic ward _n = 1
	c (n = 1)	<i>bla</i> _{VIM} (n = 1)	2021 _n = 1	Urology _n = 1
	d** (n = 1)	<i>bla</i> _{VIM} (n = 1)	2021 _n = 1	Urology _n = 1
<i>E. coli</i>	A (n = 2)	<i>bla</i> _{KPC} + <i>bla</i> _{OXA-48} (n = 2)	2021 _n = 2	Surgery _n = 1; ICU _n = 1

Abbreviations: ICU, Intensive Care Unit; INU, Intensive Neurology Unit; IPU, Intensive Pediatric Unit; * isolate from the hospital environment; ** ERIC profiles identical with the ERIC profiles of previously isolated *E. cloacae*, representatives of two dominant hospital clones from the period 2014–2017.

DISCUSSION

The University Hospital “Saint Marina” is a 1380 bed-hospital in Varna city, located in the Northeastern Bulgaria. The hospital provides healthcare for 1 500 000 population. During the COVID-19 pandemic (2020–2021), nineteen COVID departments were structured (including two ICUs) and more than 10 500 patients were treated. In the recent years the representatives of *Enterobacteriaceae* (*K. pneumoniae*, *E. coli*, *E. cloacae* complex) are the most frequently isolated bacterial species from various clinical samples, collected from patients in the hospital. During the period 2015–2021, a significant trend of increasing the rate of CR *K. pneumoniae* was detected, ranging from 0.7% in 2015 to 9% and 19% in 2020 and 2021, respectively. During the same period only single isolates of CR *E. coli* and *E. cloacae* complex were identified, especially during the last two years (2020–2021). Possible reasons for such dramatic increase in the rate of CR *Enterobacteriaceae* are the overloading of the hospital and the medical staff during the two years of pandemic, the high number of critically ill patients and the increased use of empirical antimicrobial therapy for a long period of time. Data published by Cuntrò demonstrated no significant increase in the isolation of carbapenemase-producing *Enterobacteriaceae* in patients with COVID-19 at the beginning of the pandemic in comparison to previous years [16]. Comparing COVID-19 patients and non-COVID-19 patients in the pre-pandemic year in Serbia, Despotovic reported a statistically significant difference in the carbapenem resistance: 56.8% and 61.1% for imipenem

and meropenem in COVID-19 patients versus 24.5% and 24.3% for non-COVID-19 patients [17]. In the later periods of the pandemic an increase in the rate of these pathogens was observed in many regions of the world [8, 18]. In 2020 an increase in the colonization/infection rate with NDM-producing *Enterobacteriaceae* in a cohort of COVID-19 patients in an Italian university hospital compared to other patients was reported by Porretta. This author also found a significant increase in the duration of hospital stay, associated with these co-infections [18]. In another Italian hospital, the incidence of acquisition of KPC-producing *K. pneumoniae* in patients with COVID-19 from the ICUs increased from 6.7% in 2019 to 50% in 2020 [19].

In our study a total of 143 CR *Enterobacteriaceae* isolates were recovered from patients admitted to COVID-19 wards during the period 2020–2021, with *K. pneumoniae* being the dominant bacterial species (94.4%). Similar results were reported in a multicenter observational Italian study [9]. In the whole tested group of 45 CR isolates, we documented *bla*_{KPC} as the main gene encoding carbapenemases (60.0%), confirming the findings from Romania (44.4%), Brazil (92.3%) and Italy (52–100%) [7, 9, 19, 20, 21]. This result also correlates with the data reported by Dabrowska et al. in their extensive review covering the available literature on CR *K. pneumoniae*, associated with COVID-19 patients [22].

In addition, genes, encoding metallo-beta-lactamases (MBL) were detected in 65.9% of *K. pneumoniae* and *E. cloacae* complex isolates in our study (*bla*_{NDM}, 31.7%; *bla*_{VIM}, 34.2%). Similarly, Falcone et al. reported 43.9% MBL-producing enteric bacteria, obtained from hospitalized



COVID-19 patients [9]. The high proportion of MBL producers in the studied collection of isolates could explain the detected high rates of ceftazidime/avibactam resistance in the whole *Enterobacteriaceae* group (60%).

In our study, genes, encoding OXA-carbapenemases (*bla*_{OXA-48}) were identified only in single *E. coli* isolates (4.4%), confirming published results from Romania (11.1%) and Italy (4.8%) [9, 20].

A similar distribution of carbapenemase genes, mainly *bla*_{KPC} in *K. pneumoniae*, *bla*_{OXA-48} in *E. coli* and *bla*_{NDM} in *E. cloacae* complex, was reported by Assis et al. [23]. A prevalence of KPC, OXA-48 and VIM carbapenemases among *Enterobacteriaceae* isolates was also found by Pintado et al. in their study from 2021 [24]. Studies from Italy, France and the USA also demonstrated predominance of KPC and NDM carbapenemases in COVID-19 patients [8, 19, 21, 25, 26].

The increased isolation of CR *Enterobacteriaceae* during the COVID-19 period in our hospital is a worrying finding, which underlines the necessity of elucidating the molecular epidemiology of the circulating MDR nosocomial isolates. The results from the present study illustrate the successful intrahospital dissemination and persistence of clonally related KPC, VIM and NDM producing *K. pneumoniae* isolates, representatives of two major cluster groups, but also emergence of CR *K. pneumoniae* with unique ERIC profiles, carrying *bla*_{KPC}, *bla*_{NDM} and *bla*_{VIM} in 10 hospital wards during the same period. The isolation of NDM-producing *K. pneumoniae* from the hospital environment, demonstrates the importance of the contaminated environment as a source to acquire and further disseminate the nosocomial pathogen. Intrahospital clonal dissemination of CR *K. pneumoniae* isolates, affecting problematic patients, including patients with COVID-19, has been reported in Europe and the USA [25–29]. Similarly, nosocomial spreading of NDM-1 and OXA-48 producing *K. pneumoniae* during the COVID-19 pandemic was documented in France and Spain [30, 31].

Among the studied *E. cloacae* complex isolates in this study, all *bla*_{VIM} positive, no clonality was found. However, the identification of two of these isolates as representatives of two ESBL producing *E. cloacae* complex clones with widespread intrahospital dissemination during the period 2014–2017, is an indication for the potential of these clones to persist in the hospital over long period of time [32]. Similar to our finding, a clonal dissemination of CR *E. cloacae* complex during the COVID-19 pandemic was reported by different authors: VIM-4 and NDM-1 producing *E. cloacae* complex in an intensive care unit in an Italian hospital, NDM-1 producing *E. cloacae* in ICU in France and the USA [28, 29, 31].

CONCLUSION

The carbapenem resistance in the studied collection of isolates is mediated mainly by *bla*_{KPC}, but also by *bla*_{VIM} and *bla*_{NDM} genes. During the COVID-19 pandemic intra-

hospital dissemination of CR *K. pneumoniae*, producing carbapenemases of different molecular classes, as well as continuing circulation of dominant hospital clones of MDR *E. cloacae* complex, identified in the period 2014–2017, but already carrying carbapenemase genes, were found. This study confirms the wide on-going distribution of KPC, NDM, VIM and OXA-48 carbapenemases in *Enterobacteriaceae*, facilitated by the antimicrobial overconsumption and selective pressure during COVID-19 pandemic period. The identified persistence of endemic MDR clones necessitates continuous epidemiological investigation as an important part of the hospital infection control program.

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Declaration of competing interests: All authors declare no competing interests.

Ethical conduct of research: All studies in the presented manuscript are part of the routine diagnostic and treatment procedures in the hospital. The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Ethical Committee of Varna Medical University (124/06.01.2023).

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