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Original article

MOLECULAR EPIDEMIOLOGY OF MULTIDRUG RESISTANT *ENTEROBACTER CLOACAE* BLOOD ISOLATES FROM A UNIVERSITY HOSPITAL

Dobromira Dimitrova[1], Temenuga Stoeva[1], Rumyana Markovska[2], Petya Stankova[2], Kalina Mihova[3], Radka Kaneva[3], Ivan Mitov[2]
1) Department of Microbiology and Virology, University Hospital Saint Marina, Medical University, Varna, Bulgaria
2) Department of Medical Microbiology, Medical University, Sofia, Bulgaria
3) Molecular Medicine Center, Medical University, Sofia, Bulgaria.

ABSTRACT

Purpose: to evaluate the epidemiological relationship between 3rd generation cephalosporin resistant *Enterobacter cloacae* blood isolates collected from patients in the University Hospital in Varna city during the period March 2014 and January 2017 and to characterize the ESBLs production in these isolates.

Materials and methods: a total of 47 consecutive (nonduplicate) 3rd generation cephalosporin resistant isolates of *Enterobacter cloacae*, obtained from blood samples of patients admitted in different wards in Varna University Hospital, were investigated. Antimicrobial susceptibility to set of antimicrobial agents was tested by disc diffusion method and Phoenix (BD), and the results were interpreted according to EUCAST guidelines 2017. Identification of ESBL encoding genes was performed by PCR and sequencing. Isolates were genotyped by ERIC PCR.

Results: The antimicrobial susceptibility in the whole collection of isolates, shown in decreasing order, is as follows: amikacin, 97.8% < levofloxacin, 76.6% < trimethoprime/ sulphometoxazole, 40.4% < ciprofloxacin, 19% < gentamicin, 8.4% < cefepime, 4.2% < piperacillin/ tazobactam, tobramycin, 2.1%. Multidrug resistance was detected in 70.2% of the isolates. The most widespread enzyme was CTX-M-15, found in 95.5% (n=43). Nine different ERIC types were detected. The dendrogram of similarity revealed three main clones of *E. cloacae*: Clone I, comprising two closely related subclones (ERIC type A and Aa) (similarity coefficient 0.92), was predominant, detected in Haematology (n=9), Haemodialysis (n=8), ICU (n=6), Cardio surgery (n=3), Pulmonology (n=4) and Gastroenterology (n=1); Clones II (ERIC type C) and III were presented by 5, and 3 isolates with identical profiles, obtained from patients, hospitalized in different wards. The ERIC profiles K, L, M and P, were found in single isolates only and were interpreted as sporadic.

Conclusions: multi-drug resistance in *E. cloacae* was associated with successful intrahospital dissemination of three CTX-M-15 producing *E. cloacae* clones. Clone I was predominant, demonstrating high cross-transmission, epidemic and invasive potential. *Bla*$_{CTX-M-15}$ was identified as a major mechanism of resistance to 3rd generation cephalosporins in *E. cloacae.*

Keywords: Enterobacter cloacae, ESBL genes, epidemiology, epidemic clones,

INTRODUCTION

Enterobacter cloacae is increasingly recognized as an opportunistic pathogen responsible for a wide range of nosocomial infections (incl. hospital outbreaks) such as bacteremia, pneumonia, urinary tract and wound infections and infections of the central nervous system, particularly affecting patients in the ICUs. Increased length of hospital stay, prior administration of antibiotics (especially 3rd generation cephalosporins), the use of central venous and arterial catheters, intubation and other invasive manipulations associated with disruption of the natural mechanical barriers, increased severity of illness (diabetes, onco-hematological diseases, solid tumors, transplantation, neutropenia) are documented risk factors for acquisition of *E. cloacae* infections, especially bloodstream infections (BSI) [1, 2]. The treatment of *Enterobacter cloacae* infections can be problematic because of the increasing levels of resistance to different antimicrobial agents in this bacterial species especially to quinolones, aminoglycosides and beta-lactams, the last usually associated with the production of ESBLs and AmpC enzymes. Recently a WHO expert group defined a Priority Pathogen List for research and development of new antimicrobials active against multidrug- and extensively drug-resistant Gram-negative bacteria [3]. Third generation cephalosporin resistant *Enterobacter* spp. were included in the first priority tier, named “Priority 1: Critical”, which shows the significance and the burden of infections, these antibiotic-resistant bacteria are associated with.

E. cloacae is one of the most frequently isolated organisms from blood cultures, collected from patients, hospitalized in the University Hospital “Saint Marina” – Varna, Bulgaria in recent years. This bacterial species takes the second and third position in the etiological spectrum of BSIs

in the hospital during the period 2014 – 2016. The rate of 3rd generation cephalosporin resistant invasive isolates of *E. cloacae* during the same period shows a constant trend to increase, reaching 85% in 2017 (data not published).

The aim of this study was to evaluate the epidemiological relationship between third generation cephalosporin resistant *Enterobacter cloacae* blood isolates collected from patients in the University Hospital during the period March 2014 - January 2017 and to characterize the ESBLs production in these isolates.

MATERIALS AND METHODS

Bacterial isolates

Between March 2014 and January 2017, a total of 47 consecutive (nonduplicate) 3rd generation cephalosporin resistant isolates of *Enterobacter cloacae*, obtained from blood samples of patients admitted in different wards in Varna University Hospital, were investigated. Double - disc synergy test (DDST) with discs ceftazidime, cefotaxime, cefepime and amoxicillin/clavulanic acid in a distance of 20 mm was used to determine the production of ESBLs. Species identification was made by conventional, semi-automated (Crystal, BD) and automated systems (Phoenix, BD).

Antimicrobial susceptibility testing

Susceptibility to piperacillin/tazobactam (TZP), ceftazidime (CAZ), meropenem (MEM), gentamicin (G), amikacin (AK), tobramycin (TOBR), ciprofloxacin (CIP), levofloxacin (LVX), trimethoprime/sulfamethoxazole (T/S) was tested by disc-diffusion method and / or automated system Phoenix 100, BD and the results were interpreted according to EUCAST, 2017 guidelines [4].

Detection of ESBL encoding genes

The detection of ESBL encoding genes (bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$) was performed by PCR [5]. Eighteen representative ESBLs producing *E. cloacae* isolates, selected according to their antimicrobial resistance profile and ERIC type were chosen for sequencing of their ESBL genes [5].

Molecular typing

The clonal relatedness of the isolates was determined by ERIC PCR as previously described [5].

RESULTS

During the study period, forty-seven 3rd generation cephalosporin resistant *E. cloacae* isolates were collected consecutively from 47 patients, hospitalized in 12 clinical wards of the hospital (Table 1). All isolates were obtained from blood samples: eight were associated with bacteremia in ICU patients and thirty-nine – with bacteremia in non-ICU patients. Patient data including hospital ward and date of isolation are shown in Table 1.

Table 1. Characteristics of *Enterobacter cloacae* isolates analyzed in this study.

isolate	Date of isolation	Department	ERIC type	Resistance pattern	ESBLs type
1	10.11.2014	Haemodialysis	A	TZP, CAZ, FEP, G, TOBR, CIP, LVX, T/S	CTX-M-15
1063	25.06.2015	Haemodialysis	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1065	20.06.2015	Haemodialysis	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1068	22.06.2015	Haemodialysis	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1069	23.06.2015	Haemodialysis	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1071	22.06.2015	Haemodialysis	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1072	23.06.2015	Haemodialysis	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1081	27.06.2015	Haemodialysis	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1067	02.07.2015	Nephrology	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1062	03.07.2015	Nephrology	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
1070	02.07.2015	Nephrology	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
10	02.11.2015	Haematology	A	TZP, CAZ, FEP, G, TOBR	CTX-M-15
16	11.12.2015	Haematology	K	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
43	07.03.2016	Haematology	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
48	06.04.2016	Haematology	C	TZP, CAZ, FEP, G, TOBR, CIP, LVX, T/S	CTX-M-15
62	20.04.2016	Haematology	F	TZP, CAZ, FEP, G, TOBR, CIP, LVX, T/S	CTX-M-15
103	01.12.2016	Haematology	Ab	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
108	11.12.2016	Haematology	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
112	05.01.2017	Haematology	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
116	15.01.2017	Haematology	A	TZP, CAZ, FEP, G, TOBR	CTX-M-15
123	18.01.2017	Haematology	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15

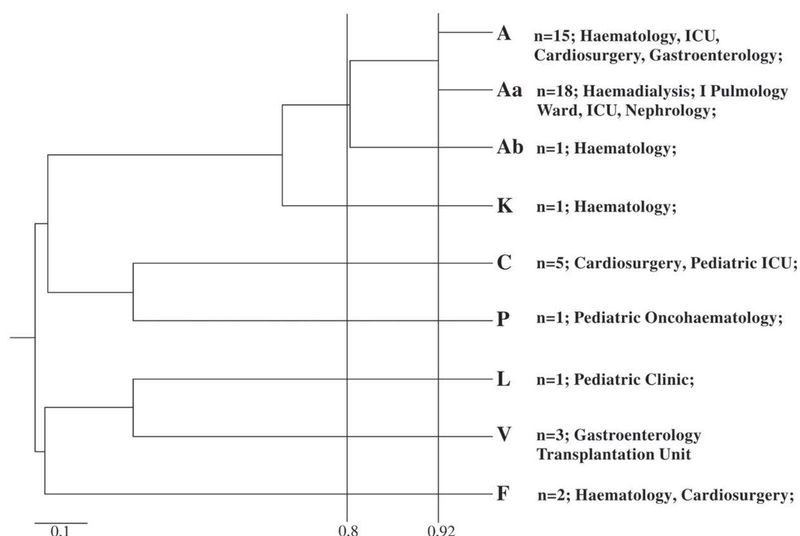
126	19.01.2017	Haematology	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
128	26.01.2017	Haematology	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
5	13.11.2015	Transpalantation Department	V	TZP, CAZ, FEP, G, TOBR, CIP, LVX	CTX-M-15
7	30.11.2015	Cardiosurgery	C	TZP, CAZ, FEP, TOBR, CIP	CTX-M-15
41	24.03.2016	Cardiosurgery	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
73	20.06.2016	Cardiosurgery	F	TZP, CAZ, FEP, TOBR, CIP	CTX-M-15
119	15.01.2017	Cardiosurgery	A	TZP, CAZ, FEP, G, TOBR, T/S	CTX-M-15
694	08.04.2014	Cardiosurgery	Aa	TZP, CAZ, FEP, G, TOBR, CIP, T/S	not detected
727	21.05.2014	Cardiosurgery	C	TZP, CAZ, FEP, G, TOBR, CIP, LVX, T/S	CTX-M-15
107	10.12.2016	I Cardio Department	C	TZP, CAZ, CIP, LVX	not detected
85	12.09.2016	ICU	A	CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
90	24.08.2016	ICU	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
105	06.12.2016	ICU	A	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15
125	19.01.2017	ICU	V	TZP, CAZ, FEP, G, TOBR, CIP, LVX	CTX-M-15
129	27.01.2017	ICU	Aa	TZP, CAZ, FEP, G, TOBR	CTX-M-15
668	07.03.2014	ICU	Aa	TZP, CAZ, FEP, G, AK, TOBR, CIP, LVX, T/S	CTX-M-15
706	11.04.2014	ICU	Aa	TZP, CAZ, FEP, G, TOBR, CIP, LVX, T/S	CTX-M-15
739	09.06.2014	I Pulmonology	Aa	TZP, CAZ, FEP, G, TOBR, T/S	CTX-M-15
744	13.06.2014	I Pulmonology	Aa	TZP, CAZ, FEP, G, TOBR, T/S	CTX-M-15
745	13.06.2014	I Pulmonology	Aa	TZP, CAZ, FEP, G, TOBR, T/S	CTX-M-15
746	13.06.2014	I Pulmonology	Aa	TZP, CAZ, FEP, G, TOBR, T/S	CTX-M-15
109	12.12.2016	Gastroenterology	V	TZP, CAZ, FEP, G, TOBR, CIP, LVX	CTX-M-15
115	20.01.2017	Gastroenterology	A	TZP, CAZ, FEP, G, TOBR, CIP	CTX-M-15
67	13.05.2016	I Pediatric Clinic	L	TZP, CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-3
133	06.02.2017	Pediatric Haematology	P	CAZ, TOBR, T/S	SHV-12
1073	27.06.2015	Pediatric ICU	C	CAZ, FEP, G, TOBR, CIP, T/S	CTX-M-15

The antimicrobial susceptibility in the whole collection of isolates (n=47), shown in decreasing order, is as follows: amikacin, 97.8% < levofloxacin, 76.6% < trimethoprime/ sulphometoxazole, 40.4% < ciprofloxacin, 19% < gentamicin, 8.4% < cefepime, 4.2% < piperacillin/ tazobactam, tobramycin, 2.1%. Meropenem demonstrated fully preserved activity (no resistance detected). Most of the isolates (70.2%) exhibited a multidrug resistant phenotype characterized by resistance to all tested β-lactams, aminoglycosides and fluoroquinolones. The screening DDST, performed with all forty-seven 3rd generation cephalosporin resistant isolates, was positive in 45 isolates, suggesting ESBLs production. Two isolates did not give a positive reaction in this test. ESBL-group specific PCR detected the presence of $bla_{\text{CTX-M}}$ and bla_{SHV} genes in forty-four, and one isolates, respectively. The sequence analysis proved the presence of $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$ and $bla_{\text{SHV-12}}$. The overall rate of CTX-M enzymes was 97.7% (n=44). The most widespread enzyme was CTX-M-15, found in 95.5% (n=43). CTX-M-3 and SHV-12 ESBLs were identified in single isolates only. No ESBLs genes were found in two isolates that demonstrated resistance to ceftazidime and cefotaxime, a result that suggested a possible hyper production of AmpC enzyme.

All studied isolates were genotyped by ERIC - PCR. Nine different types, designated as ERIC A, Aa, Ab, C, F, K, L, M and V were detected (table 1, figure 1). The dendrogram of similarity revealed three main clones of *E. cloacae* (clones I - III). Clone I, comprising two closely related subclones (ERIC type A and Aa) with 15 and 18 isolates respectively (similarity coefficient 0.92), was predominant (figure 1). The majority of subclone A isolates were recovered in Haematology (n=9) between November 2015 and January 2017, in Haemodialysis (n=8) predominantly in June 2015 and in the ICU (n=6) between March 2014 and January 2017. In addition type A/Aa isolates were also detected in Cardio surgery (n=3) in April 2014, March 2016 and January 2017, in Pulmonology (n=4) in June 2014, Nephrology (n=3) - June 2015 and Gastroenterology (n=1) - in January 2017 (table 1). ERIC type A isolates were as-

sociated predominantly with Haematology and ICU, while type Aa isolates - with Haemodyalisis and Pulmonology. The first type Aa isolate was detected in March 2014 in the ICU, followed by an identical isolate in Cardio surgery in April 2014. The closely related type A was identified for the first time in November 2014 in Haemodyalisis. All isolates within clone I were resistant to 3rd generation cephalosporins, and this was associated with CTX-M-15 production. Clone II (ERIC type C) was presented by 5 isolates with identical profiles, obtained from patients in pediatric ICU, Cardio Surgery, Cardiology ward and Haematology during the period of May 2014 and December 2016. They all were resistant to 3rd generation cephalosporins, owing to the production of CTX-M-15 for 4 of them and probable hyperproducton of AmpC in one isolate, which was negative for ESBL genes. Clone III (ERIC type V) is presented by three identical isolates from Gastroenterology, ICU and Transplantation Department and was associated also with CTX-M-15 production. The ERIC profiles K, L, M and P, were found in single isolates only and were interpreted as sporadic. L and P type *E. cloacae* were associated with CTX-M-3 and SHV-12 production respectively. The isolate distribution among the wards and their respective ERIC type are shown in Table 1.

Fig. 1. Dendrogram of similarity showing relatedness between the ERIC profiles of 47 *Enterobacter cloacae* isolates.



DISCUSSION

This study provides epidemiological data on 3rd generation cephalosporins resistant *E. cloacae* isolated during the period March 2014 – January 2017 from blood cultures, collected from patients hospitalized in Varna University Hospital, Bulgaria. Over the past decades, *E. cloacae* have become the third most frequent and lethal *Enterobacterales* species involved in BSIs [6, 7, 8]. Even more, ESBLs and MDR isolates of *E. cloacae* have been reported from different parts of the world, causing difficult to treat infections (incl. BSI). Most of these problematic isolates are associated with the production of AmpC enzymes (chromosomal or plasmid) and/or plasmid encoded ESBLs. The ESBLs in this bacterial species, conferring resistance to third and fourth generation cephalosporins, are widely disseminated, and the most common enzymes belong to CTX-M, SHV and TEM types. The CTX-M enzymes dominate in Europe and Latin America, while SHV types, especially SHV-12 – in Asia [9]. *Bla*$_{CTX-M-15}$ is the most common gene associated with resistance to 3rd generation cephalosporins (ceftazidime, cefotaxime), detected worldwide in clinically important Gram-negative bacteria, especially *E. coli* and *K. pneumoniae* [10, 11]. It is not surprising that other members of *Enterobacterales* such as *Enterobacter cloacae* sharing similar ecological niches with *E. coli* and *K. pneumoniae* could acquire *bla*$_{CTX-M-15}$, which is frequently linked to epidemic IncF plasmids [12].

E. cloacae is the sixth most commonly isolated organism in Varna University Hospital. The level of 3rd generation cephalosporin resistance among *E. cloacae* in our hospital during the period 2014 – 2016 is 56%. During the same period, the resistance in *E. coli* and *K. pneumoniae* against 3rd generation cephalosporins is 18.4% and 59.4% respectively. Among all 3rd generation cephalosporin resistant isolates included in the current study, 95.7% (45/47) were ESBL positive. The resistance to ceftazidime and cefotaxime was associated predominantly with CTX-M-15 in 95.5% and only in single isolates with SHV-12 and CTX-M-3 ESBLs. Similar to our results, studies performed in French, Greek, German and Spanish hospitals during the last decade also report a significant increase of clinical isolates of 3rd generation cephalosporin resistant *E. cloacae,* associated with ESBLs production [13]. Our previous study in Varna University Hospital in 2011 also detected a high rate of CTX-M-15 producing *E. cloacae*, but a high proportion of CTX-M-3 and SHV-12 producers was found too. A co-production of CTX-M-15/CTX-M-3 and SHV-12 was also detected during this earlier period [14]. The prevalence of *bla*CTX-M-15 in our current study is much higher than the results of the National French Reference Center, which reported dominance of CTX-M-15 (47%), followed by SHV-12 enzyme (34%) among 200 clinical isolates of *E. cloacae* [15]. Sounna et al. also found a high prevalence of CTX-M (CTX-M-15 and CTX-M-3) ESBLs (69%) and to lesser extend SHV-12 enzyme [16]. Livermore reported a prevalence of CTX-M enzyme group 9 (CTX-M-9 and CTX-M-14) in Spain but prevalance of CTX-M enzyme group 1 (CTX-M-15 and CTX-M-3) in the other European countries [17]. Among the collection of 195 rectal carriage *E. cloacae*

isolates resistant to 3rd generation cephalosporins, collected from patients in 12 hospitals across Europe and Israel, CTX-M-15 was the most frequent ESBL (60.4%), followed by SHVs (33.3%) and TEMs (2.1%) [18]. BSIs, caused by CTX-M-15 producers of *E. cloacae*, were reported by Ho et al. [19]. Seki et al. identified 42% of the bloodstream isolates of *E. cloacae* in Brasil as ESBL positive, with CTX-M-15 being the most common type [20]. In 2011 Mshan et al. described an outbreak of neonatal sepsis caused by *E. cloacae* carrying *bla*CTX-M-15 in a neonatal unit of a tertiary care hospital in Tanzania [21]. In contrast, the SHV-12 enzyme predominates in ESBL producing *E. cloacae* bloodstream isolates from China and Taiwan [2, 9]. The prevalence of ESBLs producing bloodstream *E. cloacae* isolates is diverse and varies according to the geographic region: from 5-10% for Europe and USA, 15% for China to 42% and 50% for Brasil and South Korea respectively [9, 22, 23, 24].

The results from antimicrobial susceptibility testing in this study showed that most of the ESBL isolates demonstrated multidrug resistance phenotype (MDR) with highest resistance rates to tobramycin (98%), gentamicin (91.6%), ciprofloxacin (81%) and trimethoprime/ sulphometoxazole (59.6%). The common association of resistance to 3rd generation cephalsporins with resistance to other antimicrobial agents and MDR profile can be explained with the plasmid location of the ESBL genes. Different types of plasmids (IncFI, IncFII, Inc A/C, Inc M/L, IncHI2) have been identified to play an important role in the dissemination of ESBL genes. Some of these plasmids, in addition to the ESBLs genes, carry also genes that encode resistance to other groups of antimicrobial agents, thus conferring MDR phenotype. These plasmids, known as "epidemic resistance plasmids", have been previously detected in *Enterobacterales* of different origin and sources [25, 26]. Plasmids from IncL/M and IncFII types were identified in our previous study on ESBL producing *Enterobacter* spp., *Pantoea agglomerans* and *Serratia marcescens* and were found to be associated with *bla*CTX-M-3 and *bla*CTX-M-15 respectively [14]. The results from this study, demonstrating the high level of MDR among the studied bloodstream isolates, are in concordance with the findings of the European Antimicrobial Resistance Surveillance Network (EARS-Net) for invasive *E. coli* and *K. pneumoniae* isolates, collected in 29 European countries during 2016. In its annual surveillance document on antimicrobial resistance in Europe, EARS Net reported that the combined resistance in invasive isolates (from blood and cerebro-spinal fluid), measured as resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides, increased significantly for the period 2013 – 2016, with the highest levels of 22% for *E. coli* and 55.7% for *K. pneumoniae*, detected in Bulgaria and Slovakia respectively. The combined resistance in the included Bulgarian *K. pneumoniae* isolates also was among the highest levels reported from all 29 European countries: 45.9% [27].

No data on molecular epidemiology of *E. cloacae*, associated with BSI are available in Bulgaria. One of the major scopes of this study was to investigate the presence of *bla* genes, mediating the resistance to 3rd generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) in *E. cloacae* blood isolates and their association with particular clones. In recent years *E. cloacae* has emerged as a major nosocomial pathogen causing hospital acquired BSI worldwide. Many authors describe hospital outbreaks often associated with MDR *E. cloacae* isolates and their dissemination in the hospital environment [1, 28, 29, 30, 31, 32]. The outbreaks of *Enterobacter* infections have been frequently traced to contaminated iv products, blood products, distilled water and pressure monitoring devices [33, 34]. In some hospitals hand, washing sinks and endoscopes have been described to be putative sources of clonal *E. cloacae* outbreaks [34].

In our study, ERIC PCR identified the existence of a clone (clone I), consisting of two closely related (0.92) CTX-M-15 producing *E. cloacae* subclones (A and Aa), presented by isolates with similar profiles. Originating in 2014, these isolates were found to persist until January 2017. Although the first isolate of subclone Aa was recovered in March 2014 in the ICU, it was predominant as clustered cases of bacteremia in the Pulmonology ward in June 2014, in Hemodialysis and Nephrology in June – July 2015. As single isolates, it was recovered in Cardio surgery (April 2014) and ICU (March 2014 and January 2017). The first isolate of subclone A was recovered in November 2015 in Hemodialysis. Single isolates, representing subclone A were found in Cardio Surgery (March 2016), ICU (August 2016; September 2016, December 2016), but clustered cases of bacteremia, caused by subclone A were identified in January 2017 in patients hospitalized in four different wards of the Hospital: Hematology, Cardio Surgery, Cardiology Department and ICU. The detection of isolates with identical ERIC profiles, obtained from patients, hospitalized in different wards and the temporal distribution of the cases over 35 months indicated nosocomial intermittent cross-transmission of a human or environmental source. Clone I was represented by isolates recovered mainly in Hemodialysis, Hematology and ICU in our hospital during 35 months period. The longtime this cluster presented in the hospital demonstrates its high capacity to persist. Nosocomial acquisition and dissemination due to contaminated environmental sources (one or multiple reservoirs) and hands of the medical stuff (HMS) can be suggested, although the microbiological investigation failed to detect fecal carriage of *E. cloacae* or to identify *E. cloacae* in the hospital environment. In addition, the association with cases of nosocomial bacteremia is an indication for the invasive potential of the closely related subclones A and Aa. They consisted of MDR isolates carrying the *bla*CTX-M-15 gene. The results clearly demonstrate that the intrahospital dissemination of these two subclones contributes to the emergence of CTX-M-15 producing *E. cloacae* in the hos-

pital. Outbreaks of *E. cloacae* producing CTX-M-15 have been reported from different parts of the world – Spain, Belgium, France, Greece, Japan, Tansania [12, 34, 35, 36]. The major clone I (A and Aa) in this study persisted almost three years. Similar to this result, R. Beyronthy reported VIM-4 producing *E. cloacae*, associated with a nosocomial outbreak in 2016 in France that persisted 1 year despite the application of specific isolation precautions for patients or infected persons. The epidemic clone was identified as ST873 *E. cloacae* and an enhanced biofilm formation in the epidemic strain was found, which may explain its successful persistence. The same authors also investigated another nosocomial outbreak caused by the same ST873 *E. cloacae*, but associated with CTX-M-15 production [34]. In addition to ST873, phylogenetic studies on MDR *E. cloacae* in the recent years identify other high risk successful International clones (ST45, ST66, ST74, ST78, ST114, ST108) associated with ESBLs and/or carbapenemase producers and with epidemic and even pandemic potential, similar to some *E. coli* and *K. pneumoniae* clones [18, 37]. Further studies are required to identify if the clones, identified in the current study are local or represent one of these International clones.

Similar to clone I, two smaller clones were identified - clone II and clone III, represented by identical CTX-M-15 producing isolates with ERIC type C and V, respectively. In contrast, to clone I, these isolates were associated only with single cases of bacteremia in different wards of the hospital - ICU, Cardio Surgery, Cardiology Department, Hematology, Gastroenterology and Transplantation Department.

E. cloacae isolates from the three clones identified in the current study, demonstrated MDR. Carbapenems and amikacin were the most active agents against these isolates and are the drugs of choice to treat BSIs. Unfortunately, a negative case for our hospital at the beginning of 2018 was the first isolation of a carbapenem resistant isolate of *E. cloacae* from a urine sample of a patient hospitalized in the Hematology Department with extremely limited options for antimicrobial treatment.

CONCLUSIONS

E. cloacae resistant to 3rd generation cephalosporins emerged as an important nosocomial pathogen in Varna University Hospital. During the period 2014 – 2017 multidrug resistance in *E. cloacae* was associated with successful intrahospital dissemination of three CTX-M-15 producing *E. cloacae* clones. Clone I was predominant, demonstrating high cross-transmission, epidemic and invasive potential. $\textit{Bla}_{\text{CTX-M-15}}$ was identified as a major mechanism of resistance to 3rd generation cephalosporins. Our study confirms the wide on-going distribution of CTX-M-15, CTX-M-3 and SHV-12 ESBLs in *Enterobacteriaceae* and particularly in *E. cloacae.*

Abbreviations:
TZP - piperacillin/tazobactam,
CAZ - ceftazidime,
MEM - meropenem,
G - gentamicin,
AK - amikacin,
TOBR - tobramycin,
CIP - ciprofloxacin,
LVF - levofloxacin,
T/S - trimethoprime/sulfamethoxazole.

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Address for correspondence:
Prof. Temenuga Stoeva, MD, PhD
Department of Microbiology and Virology, Medical University, Varna
55, Marin Drinov Str., Varna, Bulgaria.
E-mail: temenuga.stoeva@abv.bg