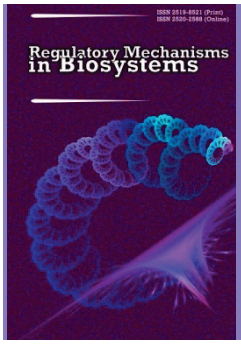

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The frequency of β-lactamase genes in ESBL-producing *Klebsiella pneumoniae* isolates in Ukraine

H. Filonenko*, V. Ishchenko**, D. Garcia***, V. Tsedyk**, V. Korniyenko**, L. Ishchenko**

**Ukrainian Children's Cardiac Center, Kyiv, Ukraine*
***National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine*
****Battelle Memorial Institute, Columbus, Ohio, USA*

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Ukrainian Children's Cardiac Center, Chornovola st., 28/1, Kyiv, 04050, Ukraine. Tel.: +38-098-233-44-29. E-mail: baclabccc@ukr.net

National University of Life and Environmental Sciences of Ukraine, Heroyiv Oborony, 15, Kyiv, 03041, Ukraine. Tel.: +38-096-577-91-66. E-mail: ischenkovd@ukr.net

Battelle Memorial Institute, King av., 505, Columbus, Ohio, 43201, USA. Tel.: +1-800-201-20-11. E-mail: garciad@battelle.org

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This study aimed to analyze the frequency of *bla*TEM, *bla*SHV, and *bla*CTX-M encoding genes in ESBL-producing *Klebsiella pneumoniae* isolates from various clinical specimens obtained from the Ukrainian Children`s Cardiac Center in Kyiv, Ukraine (2018–2021). Antibiotic susceptibility was performed by the standardized Kirby–Bauer disk diffusion method and phenotypic double-disk synergy tests were used for screening of ESBL production. Detection of *bla* genes was performed by multiplex gel-based PCR. Among the 75 samples investigated, 40 were from male patients and 35 were from females. Most of the patients were in the pediatric (n = 34) and newborns (n = 22) groups. Most *K. pneumoniae* isolates were cultured from blood (n = 22), sputum (n = 21), and the respiratory tract (n = 18). The prevalence of ESBL-producing *K. pneumoniae*, among antibiotic resistant *K. pneumoniae* was 75.6% in 2018, 84.7% in 2019, 89.3% in 2020, and 88.2% in 2021. In total, 75 ESBL-producing *K. pneumoniae* were 100.0% resistant to cefuroxime and ceftriaxone and showed high resistance to other antibiotics, including carbapenems. In total, 69 of the 75 isolates were PCR positive for one or more of these three *bla* genes. The study shows a predominance of *bla*CTX-M in the investigated strains. The prevalence of *bla*CTX-M, *bla*TEM, and *bla*SHV genes among the isolates was 84.1%, 53.6%, and 52.9%, respectively. More than half (63.8%) of the ESBL-producing *K. pneumoniae* isolates were positive for at least two ESBL genes. In addition, 26.1% of the isolates harbored all three of these *bla* genes. This study presents the prevalence of three important *bla* genes in ESBLs-producing *K. pneumoniae* and reveals a dramatic increase in the resistance of *K. pneumoniae* to the third-generation of cephalosporins in recent years. This suggests a need for more epidemiological studies, and a need to strengthen infection prevention and control measures in Ukraine.

Keywords: *Klebsiella pneumoniae*; ESBLs; *bla*TEM; *bla*SHV; *bla*CTX-M; antibiotic resistance genes.

Introduction

Klebsiella pneumoniae is a serious nosocomial pathogen that causes hospital-acquired bacterial infections such as pneumonia, urinary tract infections, septicemia, liver abscesses, and soft tissue infections (Flokas et al., 2016; Caneiras et al., 2019). The rise of extended spectrum beta-lactamase (ESBL) producing *K. pneumoniae* is a serious public health threat (Effah et al., 2020). According to the World Health Organization (WHO) third-generation cephalosporin-resistant and carbapenem-resistant *K. pneumoniae* have become a public health threat worldwide and a global priority for research and development of new antibiotics (Tacconelli et al., 2018). ESBLs first appeared in Western Europe and quickly spread to the USA and Asia. The first TEM phenotype ESBL was described in 1989 (Sougakoff et al., 1988). The SVH phenotype was described in 1985 (Huletsky et al., 1993), and the CTX-M-type β-lactamase enzymes were described in the late 1980s (Bauernfeind et al., 1990). Since the early 2000s, CTX-M-type enzymes have been defined as the dominant ESBL type that replaced TEM and SHV groups worldwide (Walther-Rasmussen & Høiby, 2004).

ESBLs are class A serine β-lactamases encoded mostly by plasmids. ESBLs confer resistance to most β-lactam antibiotics, including expanded-spectrum cephalosporins and monobactams (Shaikh et al., 2015). While expanded-spectrum β-lactam antibiotic hydrolysis and clavulanate inhibition are common biochemical characteristics of ESBLs, the genes that code these enzymes vary in sequence and divide into several families. The most common gene families are *bla*TEM, *bla*SHV, and *bla*CTX-M. The TEM- and SHV-type ESBLs are genetically similar and differ by only a few amino acids. The genetic diversity of CTX-M type ESBLs is significantly higher (Castanheira et al., 2021). Most studies show that ESBL-producing bacteria carry various combinations of these main types of *bla* genes (Empel et al., 2008; Önnberg et al., 2011; Veeraraghavan et al., 2017; Shoja et al., 2018; Caneiras et al., 2019; Dirar et al., 2020).

While the most common method for determining antimicrobial resistance (AMR) is antimicrobial susceptibility testing, molecular techniques such as PCR and whole genome sequencing (WGS) are becoming increasingly important for investigating this field. These methods are being applied more routinely for diagnostics and for the surveillance of antibiotic-resistant microorganisms (Sukhum et al., 2019; Di Cesare, 2021). However, sophisticated technologies such as whole genome sequencing are expensive and are used mainly in developed countries, whereas in low-income countries such as Ukraine, antimicrobial susceptibility testing is still the primary method used (Sulis et al., 2022).

Ukraine's latest report presented to the European Centre for Disease Prevention and Control reports the presence of third-generation cephalosporin-resistant *K. pneumoniae* in Ukraine (European Centre for Disease Prevention and Control. & World Health Organization., 2022) However, information on the molecular characterization of ESBL-producing Enterobacteriaceae isolates in Ukraine is still lacking. Understanding the antimicrobial resistance patterns and the prevalence of resistance genes among bacterial pathogens in a geographical area is important for surveil-

lance activities and disease control. This study aimed to determine the prevalence of *bla*TEM, *bla*SHV, and *bla*CTX-M encoding genes in *K. pneumoniae* isolates from various clinical specimens obtained from the Ukrainian Children's Cardiac Center in Kiev, Ukraine. Our approach was to first identify ESBL-producing *K. pneumoniae* isolates through double disk diffusion assays, followed by PCR screening of these isolates for three bla genes.

Materials and methods

Sample collection. This study included 75 non-repetitive, phenotypically positive, ESBL-producing *K. pneumoniae* isolates collected between January 2018 and December 2021 at the Ukrainian Children`s Cardiac Center in Kyiv. Clinical specimens were collected as part of routine diagnosis. During this period, 75 *K. pneumoniae* isolates from 529 isolates that were resistant to third-generation cephalosporins and were selected for this study.

Antibiotic susceptibility testing. Antibiotic susceptibility testing of *K. pneumoniae* was performed by the standardized Kirby–Bauer disk diffusion method. The testing was carried out on Mueller-Hinton agar medium against 12 antibacterial agent disks (Himedia, India). The antibacterial agents tested included cefuroxime (30 μg), ceftriaxone (30 μg), amikacin (30 μg), tigecycline (15 μg), tobramycin (10 μg), imipenem (10 μg), ertapenem (10 μg), meropenem (10 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), and ofloxacin (5 μg). Using the interpretation table based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, inhibition zone sizes of each antibiotic were interpreted, and each isolate was reported as a susceptible, standard dosing regimen (S), susceptible, increased exposure (I), and resistant (R) (European Committee on Antimicrobial Susceptibility Testing, 2017).

Screening of ESBL production. The phenotypic double-disk synergy test was conducted with susceptibility testing described by Jarlier et al. (1988). Cephalosporin inhibition zone extension toward the amoxicillin-clavulanic acid disk was interpreted as a positive indicator for ESBL production. Results were interpreted using EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing, 2017).

Screening of carbapenemase production. Bacterial isolates that showed intermediate or resistance to imipenem or meropenem were considered as suspected carbapenemase producers and were screened by the combined-disk tests containing meropenem with various inhibitors (Liofilchem, Italy). Class A carbapenemases (KPC) were inhibited with boronic acid, and class B carbapenemases with dipicolinic acid. Cloxacillin, which inhibits AmpC β lactamases, was added to the tests to differentiate between AmpC hyperproduction plus porin loss and carbapenemase production. For phenotypic confirmatory of OXA-48-like carbapenemase temocillin disks were used (van Dijk et al., 2014; European Committee on Antimicrobial Susceptibility Testing, 2017).

Identification of ESBL genes by multiplex polymerase chain reaction (PCR). DNA was extracted from isolates using the boiling method from a 24-hour cell culture (Dashti et al., 2009). The PCR mix in a total volume of 25 μL contained 2X PCR-buffer (Thermo Scientific), 2.5 mM $MgCl_2$ (Thermo Scientific), 2.5 mM dNTP mix (Thermo Scientific), 10 pmol of each primer (Table 1), (Monstein et al., 2007) 1 unit of Platinum™ Taq DNA Polymerase (Thermo Scientific) and 5 μL of DNA template. The temperature conditions included an initial denaturation step at 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. PCR products were separated on a 1.2% agarose gel (w/v) containing 0.5 μg/mL ethidium bromide.

Table 1
bla primers employed, and expected amplicon sizes

ESBL gene target		Primer sequences (5'–3')	Amplicon Size, bp
*bla*CTX-M	Forward	atgcgttatattcgcctgtg	593
	Reverse	tgctttgttattcgggccaa	
*bla*TEM	Forward	tcgccgcatacactattctcagaatga	445
	Reverse	acgctcaccggctccagatttat	
*bla*SHV	Forward	atgtgcagyaccagtaargtkatggc	747
	Reverse	tgggtraartargtsaccagaaycagcgg	

Results

Epidemiological and clinical data of study patients. Among the 75 samples investigated, 40 were from male patients and 35 were from females. Because samples were collected in the Children`s Cardiac Center, most of the patients were in the pediatric (n = 34) and newborns (n = 22) groups (Table 2). Most *K. pneumoniae* isolates were cultured from blood (n = 22), sputum (n = 21), and the respiratory tract (n = 18). From a clinical diagnosis point of view, 19 samples were obtained from patients with ventricular septal defects, 17 from patients with ischemic heart disease, and 14 from patients with coarctation of the aorta (Table 2).

Table 2
Patient and sample data

Patient characteristics, n = 75	Total
Age category	
Neonates (0–28 days)	22
Pediatric group (29 days – 18 years)	34
Adult (>18 years – 65 years)	19
Sex	
Female	35
Male	40
Biological specimen collected	
Blood	22
Sputum	21
Respiratory	18
Urine	5
Stool	3
Wound	2
Catheter central	1
Catheter peripheral	1
Broncho-alveolar lavage	1
Pleural cavity exudate	1
Diagnosis	
Ventricular septal defects	19
Ischemic heart disease	17
Coarctation of aorta	14
Transposition of great arteries	8
Patent foramen ovale	7
Complete atrial ventricular septal defect	4
Atrial septal defects	1
Partial anomalous pulmonary venous connection	1
Patent ductus arteriosus	1
Double outlet right ventricle	1
Infective endocarditis	1
Tetralogy of Fallot	1

Antibiotic susceptibility. Based on the double-disk synergy test for ESBL production, the prevalence of ESBL-producing *K. pneumoniae*, among antibiotic resistant *K. pneumoniae* was 75.6% in 2018, 84.7% in 2019, 89.3% in 2020, and 88.2% in 2021 (Fig. 1).

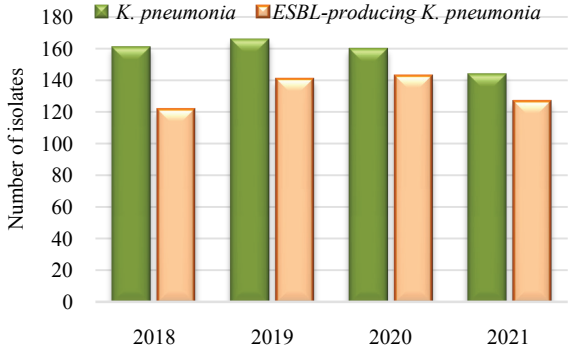

Fig. 1. Prevalence of ESBL-producing *K. pneumoniae* from 2018–2021

To study the frequency of bla genes, we selected 75 ESBL-producing *K. pneumoniae* isolates with 100.0% resistance to cefuroxime and ceftriaxone. These isolates also showed resistance to other antibiotics, including carbapenems. Only colistin was effective against the investigated isolates of *K. pneumoniae* (Table 3).

Table 3
Results of susceptibility testing of *K. pneumoniae* isolates (n = 75)

Antibiotic	%S	%I	%R
Ceftriaxone	0	0	100.0
Cefuroxime	0	0	100.0
Meropenem	16.0	4.0	80.0
Imipenem	16.0	1.3	82.7
Ertapenem	14.7	0	85.3
Tigecycline	17.3	1.3	81.3
Amikacin	18.7	0	81.3
Tobramycin	18.7	0	81.3
Ciprofloxacin	24.0	0	76.0
Levofloxacin	24.0	0	76.0
Ofloxacin	24.0	0	76.0
Colistin	97.3	0	2.7

Note: S – susceptible, standard dosing regimen, I – susceptible, increased exposure, R – resistant.

Identification of ESBL genes by multiplex PCR. Among 75 isolates tested by PCR, 69 were positive for one or more *bla* encoding genes. Typical PCR screening results of ESBL-producing *K. pneumoniae*, and other enteric bacteria are depicted in Figure 2. The prevalence of genes encoding for *bla*CTX-M, *bla*TEM, and *bla*SHV was 84.1%, 53.6%, and 52.9%, respectively (Fig. 3). Overall, 66.7% of the ESBL-producing *K. pneumoniae* isolates were positive for at least two ESBL genes.

The most frequent combination of *bla* genes in these *K. pneumoniae* isolates was *bla*CTX-M + *bla*SHV + *bla*TEM (26.1%), followed by *bla*CTX-M (23.2%), *bla*CTX-M + *bla*SHV (18.8%), *bla*CTX-M + *bla*TEM (15.9%), *bla*TEM (8.7%), *bla*SHV (4.3%) and *bla*SHV + *bla*TEM (2.9%, Fig. 4).

Discussion

ESBL-producing *K. pneumoniae* is an important pathogen responsible for a range of acute infections in hospitals. Due to high morbidity and mortality, the increasing prevalence of ESBL-producing *K. pneumonia* is a global concern (Navon-Venezia et al., 2017; Effah et al., 2020). In addition, the increased prevalence of ESBL-producing *K. pneumoniae* increases the prescribing and in turn the consumption of carbapenems, which can lead to a rise in carbapenem resistance. AMR exerts an impact across nations. However, the weight of its consequences is significantly greater in low- and middle-income countries (LMICs). The challenging issue in LMICs is poor antimicrobial stewardship implementation or absence of it, lack of resources to provide proper disinfection and sterilization in hospital and medical facilities, lack of awareness among medical staff and patients, and the ability of patients to purchase antibiotics without prescription (Pokharel et al., 2019; Lam et al., 2021). High prevalence of ESBL-producing *K. pneumoniae* is distinctive for LMICs. In a hospital-based study from Côte d'Ivoire, for example, the prevalence of ESBL-producing *K. pneumoniae* was 84% and pediatric patients were most frequently affected (Müller-Schulte et al., 2020). In Ukraine as in many LMICs, it is still common for hospitals to only conduct antimicrobial susceptibility testing when treatment with broad-spectrum antibiotics is inefficient. This can lead to increasing AMR.

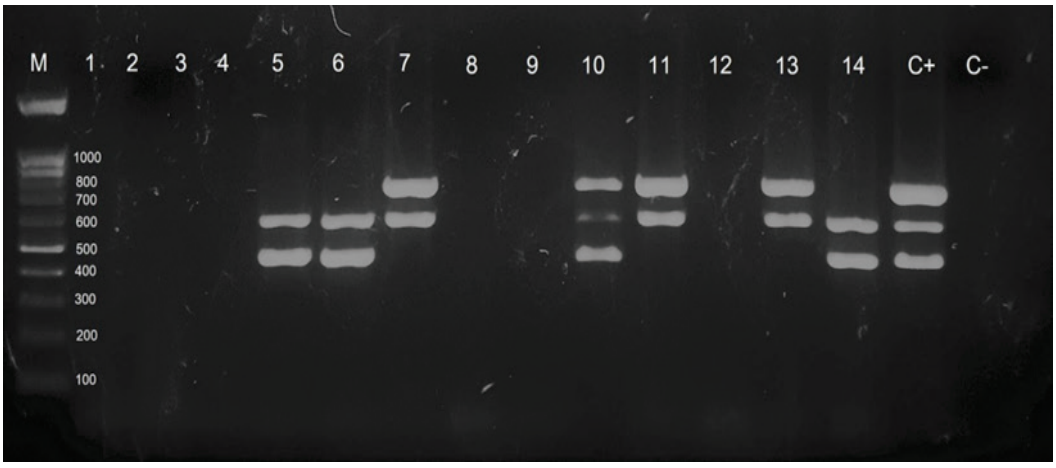

Fig. 2. PCR amplification of bla gene fragments: *bla*TEM (445 bp), *bla*CTX-M (593bp) and *bla*SHV (747bp); Lane 1 – 100 bp ladder, lane 5–7, 10–11, 13–14 clinical isolates of ESBL-producing *K. pneumoniae* lane 15 – positive control, lane 16 – negative control; Lane 2–4, 8,9 – clinical isolates ESBL-producing *E. coli* testing negative for bla genes

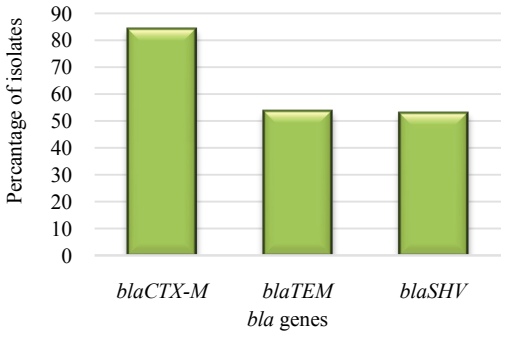

Fig. 3. Frequency of *bla* genes in ESBL-producing *K. pneumoniae* isolates (n = 69)

In this study, the prevalence of *bla* genes in ESBL-producing *K. pneumoniae* was investigated. We tested 75 ESBL-producing *K. pneumoniae* isolates that were resistant to third-generation cephalosporins. Susceptibility testing revealed that approximately 80% of these strains were also resistant to carbapenems and many other antibiotics. Multidrug resistance is not surprising because antibiotics in Ukraine were available without a prescription until 2022. This facilitated the abuse and misuse of antibiotics in the population. The prevalence of ESBL-producing *K. pneumoniae* at the Ukrainian Children`s Cardiac Center, during the investigated period, has been between 75.6% and 88.2%. In our previous study (samples collected in 2015) in the same hospital, the prevalence of ESBL-producing Enterobacteriaceae (*K. pneumoniae* was the predominant species) was only 10.9% (Filonenko et al., 2017). This suggests that there has been a dramatic increase of *K. pneumoniae* resistant to third-generation cephalosporins over recent years. The overuse of carbapenems could explain the observed resistance to them in investigated isolates of *K. pneumoniae* in our study.

The prevalence of *bla*CTX-M, *bla*SHV, *bla*TEM genes among the ESBL-producing *K. pneumoniae* isolates was 84.1%, 53.6%, and 52.9%, respectively. Our results are consistent with other studies showing, since the 2000s, that the CTX-M-type enzymes are the most common ESBL group (Empel et al., 2008; Önnberg et al., 2011). In our hospital, the pattern of *bla* genes has clearly changed since 2015, when the most prevalent gene was *bla*TEM, followed *bla*SHV and *bla*CTX-M (Filonenko et al., 2017). In general, the relative distribution of *bla* genes in ESBLs varies

across studies. In European countries, the *bla*CTX-M gene is predominant (Empel et al., 2008; Önnberg et al., 2011), whereas in Asia, the SHV gene is predominant (Feizabadi et al., 2010; Tawfik et al., 2011; Charrouf et al., 2014). Ultimately the relative distribution of *bla* genes in this study was more consistent with studies in Europe, where blaCTX-M is predominant. However, the prevalence of *bla*TEM and *bla*SHV is also high, similar to studies in Asia.

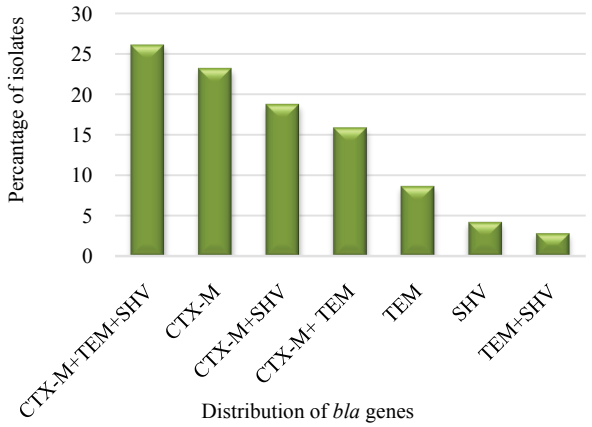

Fig. 4. Prevalence of *bla* genes in ESBL-producing *K. pneumoniae* isolates (n = 69)

We found that most investigated isolates (63.8%) harbored more than one of the *bla* genes that were screened (Fig. 3). The most frequent combination *bla* genes, among those tested, was *bla*CTX-M + *bla*SHV + *bla*TEM (26.1%), followed by *bla*CTX-M + *bla*SHV (18.8%), *bla*CTX-M + *bla*TEM (15.9%), and *bla*SHV + *bla*TEM (2.9%). The coexistence of different ESBLs is a common finding in many studies, but the frequencies of these combinations varies from region and year of investigation (Empel et al., 2008; Feizabadi et al., 2010; Shahid et al., 2011; Tawfik et al., 2011; Charrouf et al., 2014; Bajpai et al., 2017; Ceccarelli et al., 2019; Sarshar et al., 2021). However, in our hospital, there has been an increase in the frequency of strains harboring more than one *bla* gene. In our previous study, only 2 of 23 (8.7%) ESBL-producing *K. pneumoniae* isolates harbored all three of these *bla* genes (Filonenko et al., 2017). In a study from Iran, the frequency of *K. pneumoniae* with these three genes was 17.6 % (Feizabadi et al., 2010) from Saudi Arabia – 18.2% (Tawfik et al., 2011) from Lebanon – 11.74% (Charrouf et al., 2014) and from Côte d'Ivoire – 71% (Müller-Schulte et al., 2020). As suggested by Shahid et al. (2011) the presence of multiple *bla* genes in these isolates is the result of many bla encoding mobile genetic units circulating at distinct frequencies in the environment.

An important observation from this study is that 38 of the ESBL producing isolates were PCR negative for the blaSHV gene. Studies have shown that *K. pneumoniae* possess a chromosomal *bla*SHV gene that confers resistance to ampicillin, amoxicillin, carbenicillin, and ticarcillin (Chaves et al., 2001). It is the plasmid-based *bla*SHV that is expected to confer ESBL production (Hammond et al., 2008). A limitation of this study is that we were not able to perform next-generation sequencing or MALDI-TOF to distinguish *K. pneumoniae* from other species such as *K. variicola* and *K. quasipneumoniae* which were considered until recently as *K. pneumoniae* (Ohama et al., 2022; Sakai & Maesaki, 2022). These species of *Klebsiella* do not contain the chromosomal SHV gene and it could explain why many of our ESBL-producing strains were negative for *bla*SHV. In addition, three ESBL-producing *K. pneumoniae* only tested positive for the *bla*SHV gene. Because we did not determine the genetic location of the *bla*SHV gene in these 3 isolates, we can only assume that the *bla*SHV genes in these isolates are mutated genomic-based *bla*SHV genes (Hammond et al., 2008), plasmid-based, or that there are other *bla* genes (not screened in this study) responsible for ESBL production. Further studies should confirm the identity of these *Klebsiella* species and resolve the genetic location and sequences of these ESBL-related genes in Ukraine. Despite these limitations, we have shown a high prevalence of *Klebsiella* that are resistant to third-generation cephalosporins and carbapenems in a hospital facility.

It remains to be determined if patients were infected with ESBL-producing *K. pneumoniae* strains in hospital or if they had been infected before coming to the hospital. However, the high prevalence of ESBL-producing *K. pneumoniae* in our study, the high proportion of the *bla*CTX-M + *bla*SHV + *bla*TEM genotype (26.1%), and the likelihood that multiple mobile genetic units may be circulating in the hospital environment, suggest that these infections are hospital-acquired. One limitation of our study is that it was conducted in only one hospital, and the results may not reflect the patterns of *bla* genes in ESBL-producing *K. pneumoniae* in all of Ukraine. In addition, due to a lack of resources, we only investigated a subset of *K. pneumoniae* isolates that were resistant to third-generation cephalosporins.

Conclusions

Our results provide insight into the prevalence of three important *bla* genes in ESBL-producing *K. pneumoniae* in Ukraine. Importantly, this study reveals a dramatic increase in the prevalence of *K. pneumoniae* resistant to third-generation cephalosporins in recent years. More than 25% of the isolates we analyzed harbored all three of these important *bla* genes. Further studies are required to determine if these infections are hospital-acquired, but the evidence suggests it is likely. This study also suggests a need to strengthen infection prevention and control measures, to implement antimicrobial stewardship initiatives in health care facilities in Ukraine, and to strengthen laboratory capacity for antimicrobial susceptibility testing in district areas. Additionally, it is necessary to improve AMR surveillance and research programs in Ukraine.

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