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Single center study of ESBL-related strains of *Enterobacteriaceae* collected from clinical specimens of infants with the congenital heart disease using multiplex PCR amplification

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Aim. To study ESBL phenotypes, as well as to identify individual genotypes of β -lactamases and to study their prevalence among clinical strains of *Enterobacteriaceae* collected from children with congenital heart diseases. **Methods.** Clinical strains of *Enterobacteriaceae* were studied in order to determine their susceptibility to antibiotics, using an automated system, before genotyping resistance determinants using multiplex PCR. **Results.** During the period of the study, 10.9 % of ESBL-positive isolates were found among clinical strains of *Enterobacteriaceae*. Most ESBL-producing strains (33.3 %) were *Klebsiella pneumoniae*. Most strains producing extended-spectrum β -lactamases were isolated from the respiratory tract and accounted for 83.3 %. **Conclusions.** An experimental study provided new data as regards the prevalence of ESBL-producing genetic resistance determinants within the *Enterobacteriaceae* family, and their role in the development of complications. For the first time in Ukraine, we performed genotyping Multiplex PCR-based genotyping tests of ESBL-positive isolates. The study showed diagnostic value of molecular biological methods in identifying resistance determinants of microorganisms.

Keywords: antibiotic resistance, β -lactam antibiotics, *Enterobacteriaceae*, ESBL, PCR, genotyping

Introduction

Acquired antibiotic resistance of the *Enterobacteriaceae* represents globally escalating problem in treatment of healthcare-as-

sociated infections. The rapid evolution of bacterial resistance among *Enterobacteriaceae* to the beta-lactam class of antibiotics has reached epidemic proportions [1]. The background component of this growing resistance

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is the expression of enzymes known as extended-spectrum β -lactamases (ESBL) by a bacterial cell. These enzymes are generally defined as beta-lactamases and confer bacterial resistance to penicillins, first-, second-, third-generation cephalosporins and to aztreonam, but not the carbapenems and cephamycins [2].

Widespread use of these groups of antibiotics has led to the expansion of β -lactamase-encoding genes in *Enterobacteriaceae* like TEM, CTX-M, and SHV which can be encoded on mobile genetic elements, including plasmids and transposons that also often encrypt adventive resistance genes including multiple classes of antibiotics [3].

Risk factors for ESBL infection development are patient's age, prolonged in-hospital stay and previous stay at an intensive care unit (ICU) stay, prior use of antibiotics, and indwelling devices such as urinary catheters, central venous catheters, tracheostomy, and endotracheal tubes [4].

ESBL-related hospital infections are associated with negative outcomes for patients, resulting in prolonged hospital stay, increased hospitalization / inpatient costs, and increased morbidity rates [2]. The main driver of this increased morbidity is insufficient antibiotic therapy. So, early monitoring of groups of surgical patients who are at risk for infection by ESBL-producing bacteria is crucial for a choice of antibiotic treatment strategy and important point for infection spread control.

Modern data indicate a growing prevalence of ESBL's isolated from different patient groups globally [5-10]. However, neither single- nor multicenter studies have been conducted on ESBL-related hospital infections in

Ukraine. The resistance is determined by the expression of *bla* genes belonging to *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes family. To date, CTX-M enzymes are characterized as the most clinically important groups of ESBLs, which is followed by SHV- and TEM-derived ESBLs. The *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes are responsible for production of evolutionarily relative enzymes – TEM β -lactamases, SHV β -lactamases, and CTX-M β -lactamases, respectively. To date, there are about 100 derivatives of TEM-1 enzymes; 185 new β -lactamases of the TEM family have been reported worldwide, while only ninety-three variants are responsible for production of ESBLs. According to data reported recently, among one hundred seventy-two types of SHV family of enzymes, forty-five were represented as extended-spectrum beta-lactamases. The CTX-M family includes more than sixty enzymes ([http://www.eucast.org/clinical break-points](http://www.eucast.org/clinical-breakpoints)).

Multiple molecular typing methods, including PCR-based assays, have been developed for detection and identification of the growing number of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes. Though, there is still limited scientific data on epidemiology of ESBL spread in the Ukraine. To the best of our knowledge, this is the first study on the molecular epidemiology and antimicrobial susceptibilities of ESBL-producing *Enterobacteriaceae* among surgical patients in Ukraine.

The purpose of this study was to explore feasibility of simultaneous identification of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes by multiplex PCR detection in a series of clinical isolates of *Enterobacteriaceae* with previously characterized ESBL phenotype, to im-

plement routing monitoring of ESBL-producing *Enterobacteriaceae* strains at the Ukrainian Children's Cardiac Center (Kyiv, Ukraine), to determine predominant genotypes among ESBL-producing *Enterobacteriaceae* strains.

Materials and Methods

All study strains were selected based on the screening tests for the detection of ESBL-type enzymes. The isolates were collected from clinical specimens of patients hospitalized at the Ukrainian Children's Cardiac Center (Kyiv, Ukraine) during the period from January to December, 2015. The isolates were recovered from various clinical specimens, mostly tracheal discharges, throat swabs, wounds, urine and blood. The majority of the collected strains were obtained during cardiac surgery in 704 patients representing different regions of Ukraine. The average age in the studies was 128 ± 106.5 days (0 days to 1 year) (Table 1).

The ESBL phenotype of the bacterial strains was determined by the VITEK 2 GN cards and the automated identification system (*bioMérieux*, France) in accordance with internal SOPs and manufacturer's protocols.

Testing of antibiotic susceptibility and ESBL determination

Susceptibility of clinical isolates to β -lactams (ampicillin, cefazolin, cefuroxime, cefepime, ceftriaxone, imipenem, and meropenem) and to ciprofloxacin, amikacin, ofloxacin, levofloxacin, tobramycin, and trimethoprim / sulfamethoxazole was tested by using AST-N076 cards and the automated VITEK 2 system. Susceptibility was interpreted according to the

European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (<http://www.eucast.org/clinicalbreakpoints>). Thirty *Enterobacteriaceae* strains including twenty-two strains of *K. pneumoniae*, four strains of *E.coli*, and four strains of other *Enterobacteriaceae* from hospitalized patients were found to be ESBL producers. The presence of ESBL phenotype was confirmed by VITEK 2 automated system.

DNA isolation

Purified DNA from overnight bacterial cultures was isolated using the QIAGEN DNeasy Blood and Tissue Kit (Catalog# 69504) and NucleoSpin® Blood QuickPure kit (MACHEREY NAGEL, Germany). Qualitative analysis of DNA was performed by electrophoresis in 1 % agarose gel with 0.5 % TBE buffer (Tris-Borate-EDTA).

Multiplex PCR amplification

Multiplex PCR-based genotyping tests were conducted on thirty ESBL-positive isolates (Table 1) including twenty-two strains of *K. pneumoniae*, four strains of *E.coli*, and four strains of other *Enterobacteriaceae* (*Enterobacter cloacae*, *Serratia marcescens* and *Klebsiella oxytoca*).

For genotyping tests, we used sequence primers-pair and technique of multiplex PCR, with some modifications, described previously [11].

Primer-pair sequences (bla-SHV.SE/AS; TEM-164.SE and TEM-165.AS; universal CTX-M-U1/U2) used in the multiplex PCR and assay PCR amplicon sizes are given in Table 2. The reaction mixtures contained $1 \times$ PCR buffer, 200 μ M dNTP, 0.5 unit of Taq

Table 1. Overview of all strains, identified *bla* genes, and resistance phenotypes

Name of the strain	Origin	Region	Multiplex PCR			Resistance phenotype to non-β-lactam antibiotics					Active β-lactam antibiotics
			<i>bla</i> _{CTX-M}	<i>bla</i> _{TEM}	<i>bla</i> _{SHV}						
1. Kl	R	Kv		+	+	AMK ^R	TOB ^R	CIP ^R	OFX ^R		
2. Kl	w	Kv	+	+					OFX ^R	SXT ^R	MEM,IMP
3. Kl	R	Zap	+		+						MEM,IMP
4. Kl	R	Kv	+		+					SXT ^R	MEM,IMP
5. Kl	R	Lv	+	+							MEM,IMP
6. Kl	R	Vol	+	+	+	AMK ^R	TOB ^R			SXT ^R	MEM,IMP
7. Kl	R	Zh		+	+	AMK ^R	TOB ^R	CIP ^R	OFX ^R		
8. Kl	bl	Zh		+	+	AMK ^R	TOB ^R	CIP ^R	OFX ^R		
9. Kl	R	Don	+								MEM,IMP,FEP,CTM
10. Kl	R	Vol	+			AMK ^R	TOB ^R				MEM,IMP
11. Kl	R	K		+	+	AMK ^R	TOB ^R	CIP ^R	OFX ^R	SXT ^R	
12. Kl	R	Iv		+	+	AMK ^R	TOB ^R				MEM,IMP
13. Kl	bl	Dn		+	+	AMK ^R	TOB ^R	CIP ^R	OFX ^R	SXT ^R	
14. Kl	ur	Od		+	+	AMK ^R	TOB ^R	CIP ^R	OFX ^R	SXT ^R	MEM,IMP
15. Kl	oth	Vol		+		AMK ^R	TOB ^R				MEM,IMP
16. Kl	R	Vin	+	+	+						
17. Kl	R	Vin	+	+							MEM,IMP
18. Kl	R	Vol		+		AMK ^R	TOB ^R				MEM,IMP
19. Kl	R	Kv	+					CIP ^R	OFX ^R		MEM,IMP
20. Kl	R	Don		+		AMK ^R	TOB ^R				MEM,IMP
21. Kl	R	Kv		+	+						MEM,IMP
22. Kl	R	Vin	+	+							MEM,IMP
23. Kl.o	R	Zh		+	+	AMK ^R	TOB ^R	CIP ^R	OFX ^R		
24. E	R	Kv		+				CIP ^R			MEM,IMP
25. E	R	Lv		+	+			CIP ^R			MEM,IMP
26. E	R	Kv	+								MEM,IMP
27. E	R	Od		+				CIP ^R	OFX ^R		MEM,IMP
28. En	R	Lv		+	+						MEM,IMP
29. S	R	Zh	+								MEM,IMP
30. S	R	Kv									MEM,IMP,FEP,CTM

Strains: Kl: *Klebsiella pneumoniae*, Kl.o: *Klebsiella oxytoca*; E: *E. coli*; En: *Enterobacter cloacae*; S: *Serratia marcescens*, Antibiotics: AMK-amikacin, TOB-tobramycin, CIP-ciprofloxacin, OFX- ofloxacin, SXT-trimethoprim / sulfamethoxazole, MEM-meropenem, IMP-imipenem, FEP-cefepime, CTM-cefuroxime;

Origin: R - respiratory, bl - blood, ur - urine, w - wound, oth - other;

Region: Kv - Kyiv, Lv - Lviv, Od - Odessa, Don - Donetsk, Dn - Dnipro, Zh - Zhytomyr, Vol - Volyn, Vin - Vinnytsia, Zap - Zaporizhia, Iv - Ivano-Frankivsk

polymerase (Roche, Germany), 10 pmol/ μ l of each gene-specific primer and 1 μ l of DNA solution in a final volume of 10 μ l. PCR amplification conditions were as follows: initial denaturation step at 95 °C for 15 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min.

PCR amplicons were separated electrophoretically in 2 % (w/w) agarose gel containing 0.5 μ g/ml ethidium bromide (Sigma Aldrich, USA) and 0.5 % TBE buffer and visualized with «DOC-Print VX2» (Vilber Lourmat) system.

Results and Discussion

In this study, we examined the prevalence of ESBL producing *Enterobacteriaceae* and carried out molecular characterization and antimicrobial susceptibility testing of clinical samples from patients during their admission to the cardiac hospital.

ESBL-producing *Enterobacteriaceae* were obtained from clinical specimens of patients representing different regions of Ukraine (30.0 % were from Kyiv, 13.3 % were from Zhytomyr, 13.3 % were from Volyn, 10.0% were from Lviv, 10.0% were from Vinnytsa, 6.6 % were from Odessa, 6.6 % were from

Donetsk, 3.3 % were from Ivano-Frankivsk, 3.3 % were from Zaporizhia and 3.3 % were from Dnipro) hospitalized at the Ukrainian Children's Cardiac Center (Kyiv, Ukraine).

During the study period, total of 30 (10.9 %) ESBL-producing *Enterobacteriaceae* were isolated. The demographic profile of the studied population was summarized. It shows that 56.6 % (n = 17) of ESBL-positive samples were collected from male patients and 43.3 % (n = 13) of samples were from female patients (Table 1). Among the isolates, *K. pneumoniae* (33.3 %, n = 22) was prevalent ESBL-producing bacteria, ESBL-phenotype of *E. coli* accounted for 13.3 % (n = 4), and other ESBL-producing *Enterobacteriaceae* accounted for 13.3 % (n = 4). These pathogens were isolated from a variety of clinical samples, respiratory system – 83.3 % (n = 25), blood – 6.6 % (n = 2), and others – 10.0 % (n = 3).

In our study, the main ESBL producers were found to be *K. pneumoniae* and *E. coli* and other species, which is consistent with previously published results [9, 14, 15], overall part of ESBL-producing pathogens among *Enterobacteriaceae* strains was substantially lower (10.9 %) than that reported in other studies in other geographical regions [14, 15] and higher than reported in other hospital-associated infection studies [11, 13]. The majority of

Table 2. Primers used in multiplex PCR amplification

Primer name	Primer sequence (5'-3')	Amplicon size in bp.	Origin of references
bla-SHV.SE bla-SHV.AS	atgcggtatttcgcctgtg tgctttgtattcggccea	747	[11, 12]
TEM-164.SE TEM-165.AS	tcgccgcatacactatttcagaatga acgctcaccggctccagatttat	445	[11]
CTX-M-U1 CTX-M-U2	atgtgcagyaccagtaargtkatggc tgggtraartargsaccagaaycagcgg	593	[11,13]

these infections were due to *E. coli* rather than *Klebsiella pneumoniae*, as described in the non-transplant literature in many cases and as was observed in our study. However, during screening of 1065 patients at the UCLA Medical Center who had undergone heart or lung transplantation between 1996 and 2010, the incidence of ESBL-related infections in these groups appeared to be 2.2 %, 5.5 %, and 10.7 % of patients, respectively [13]. It can be explained by methodological differences in the measured prevalence levels, difference in age groups of patients and time-dependent distribution of resistance.

In this study, the respiratory tract was the major source of ESBL-producing isolates, followed by the blood and other sampling media. Similar findings have previously been reported for the ICUs at hospitals in Mexico, India and Qatar where the major source of ESBL-producing isolates were the respiratory tract

and blood [9, 16, 17]. However, in other geographical regions, urine and blood were reported as the major source of ESBL-producing bacteria [6, 12].

The antibiotic susceptibilities of ESBL-producers are presented in Table 1. Among *K. pneumoniae* strains expressing ESBL activity, high levels of resistance to ampicillin (100 %), ceftazolin (100 %), ceftriaxone (100 %), cefuroxime (100 %), and cefepime (95.7 %) were observed. Tested strains of *K. pneumoniae* were susceptible to imipenem (69.6 %) and meropenem (69.6 %). All *E. coli* ESBL-positive strains were resistant to ampicillin (intrinsic resistance), ceftazolin, cefuroxime, ceftriaxone, and cefepime. Furthermore, ESBL-positive *E. coli* strains were highly susceptible to meropenem (100 %) and imipenem (100 %). Additionally, other *Enterobacteriaceae* ESBL-positive strains were completely resistant to ampicillin, ceftazolin, and cefuroxime.

Table 3. Susceptibility of *Enterobacteriaceae* ESBL-positive strains (n=30)

Antibiotic	<i>Klebsiella pneumoniae</i>			<i>E. coli</i>			<i>Other Enterobacteriaceae</i>		
	R	I	S	R	I	S	R	I	S
AMP ampicillin	100		0	100		0	100		0
CZO ceftazolin	100		0	100		0	100		0
CXM cefuroxime	100		0	100		0	100		0
CRO ceftriaxone	100		0	100		0	50		50
FEP cefepime	95,7	4,3	0	100		0	50		50
IMP imipenem	30,4		69,6	0		100	0		100
MEM meropenem	30,4		69,6	0		100	0		100
AMK amikacin	56,5	8,7	34,8	0		100	25		75
TOB tobramycin	56,5	8,7	34,8	0		100	25		75
CIP ciprofloxacin	34,8	13	52,2	75		25	25		75
LVX levofloxacin	34,8	4,3	60,9	50		50	25		75
OFX ofloxacin	39,1		60,9	25		75	25		75
SXT trimethoprim/ sulfametoxazole	34,8	4,3	60,9	100		0	0		100

High activity against other *Enterobacteriaceae* was shown by ceftriaxone (50 %), cefepime (50 %), imipenem (100 %), and meropenem (100 %) (Table 3).

Our epidemiological data show a dramatic increase in antimicrobial resistance. Additionally, more and more studies show continuous evolution of resistance to β -lactams and other groups of antibiotics in the bacteria of the *Enterobacteriaceae* family [6–16, 18, 19].

The constant increase of simultaneous resistance to various classes of antibiotics significantly reduces the possibility of therapeutic treatment of infections caused by ESBL-producers [6–10, 12, 14, 15, 18]. That prompted us to analyze the level of resistance among ESBL-positive tested strains.

ESBL-producers revealed high resistance to antibiotic groups other than β -lactams. The results of our study are in line with some global reports [17, 20]. Our analysis showed a significant degree of ESBL-positive *K. pneumoniae* resistance to such antibiotics as ofloxacin, levofloxacin, trimethoprim/sulfamethoxazole and ciprofloxacin. The percentages of resistant strains were, respectively, 60.9 %, 60.9 %, 60.9 %, and 52.2 %. *E. coli* ESBL-positive strains appeared to be resistant to such antibiotics as ciprofloxacin (75 %), levofloxacin (25 %), and ofloxacin (25 %) (Table 3).

ESBL-positive strains showing simultaneous resistance to β -lactamases and antibiotics of other groups are defined as multidrug-resistant strains [1, 2]. Global spread of multidrug-resistant strains as an etiological factor of infections is described in numerous reports [1–21]. Characteristic location of genes responsible for resistance is the reason contributing

to the prevalence of this phenomenon. Genes encoding β -lactamases are often located in mobile genetic elements such as plasmids, allowing horizontal transfer of these genes between bacteria of *Enterobacteriaceae* species and non-fermenting bacteria [3].

In *Enterobacteriaceae*, resistant genes responsible for resistance to different groups of antibiotics are often located on the same plasmids in a close neighborhood and, thus can be transmitted at the same time to other bacteria [21].

The ESBL-producing pathogens identified by phenotypic methods were also analyzed by using PCR methods. Of 30 ESBL isolates, 6.6 % harbored multiple *bla* genes simultaneously and the prevalence of *bla*_{TEM} was as high as 70.0 %, followed by *bla*_{SHV} at 46.6 %, and *bla*_{CTX-M} at 46.6 %. The majority of the TEM-positive isolates were *K. pneumoniae* (81.8 %), *E. coli* (50.0 %), and other *Enterobacteriaceae*, (33.1 %); however, all CTX-M and SHV positive isolates were *K. pneumoniae* (50.0 % and 59.0 % respectively). Furthermore, all three *bla* genes (TEM, SHV, and CTX-M) were detected in only 9.0 % of *K. pneumoniae* isolates, while two genes (SHV/CTX-M) were present in 9.0 % of *K. pneumoniae*, with TEM/CTX-M being present in 22.5 % of *K. pneumoniae* and TEM/SHV being detected in 45.5 % of *K. pneumoniae*, and 33.3 % in other *Enterobacteriaceae* isolates (Table 1, Fig. 1).

Conclusions

Compared with previous data from other geographical regions, our study shows relatively low prevalence (10.9 %) of ESBL-producing *Enterobacteriaceae*. Though lower than in

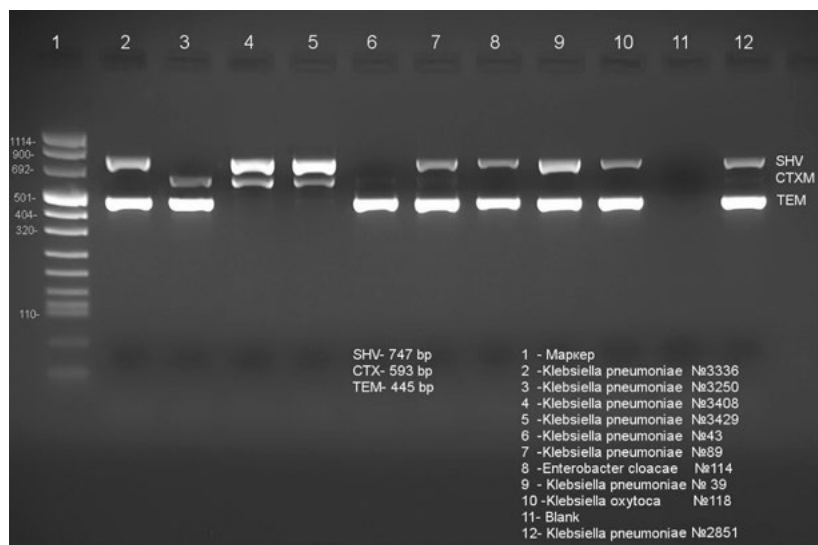


Fig. 1. Electropherogram analysis of multiplex PCR amplified products.

1 – DNA molecular weight marker; 2–7, 9 – *Klebsiella pneumoniae*; 8 – *Enterobacter cloacae*; 10 – *Klebsiella oxytoca*; 11 – blank, 12 – control strain- *Klebsiella pneumonia* ATCC 700603.

other countries and regions, our results suggest that there is sufficient global infection burden to warrant public health interventions. Notably, majority of isolates were multi-drug resistant and belonged to TEM plasmid-type. The emergence of TEM producing *Enterobacteriaceae* isolates is of major concern and necessitates further control and research in this area. As meropenem shows good activity against these ESBL producers, it should be restricted for managing patients with suspected Gram-negative bacterial infections with ESBL production. Additionally, antimicrobial control and early detection by active surveillance in combination with effective infection monitoring programs and methods are key steps for reducing or controlling the spread of ESBL-positive hospital-acquired infections in Ukraine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Дослідження штамів *Enterobacteriaceae*, що продукують БЛРС, і виділені з клінічних зразків у дітей з вродженими вадами серця з допомогою мультиплексною ПЛР.

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Мета. Вивчити фенотипи БЛРС і ідентифікацію обраних генотипів β-лактамаз і частоту їх поширення серед клінічних штамів *Enterobacteriaceae*, виділених у дітей з вродженими вадами серця. **Методи.** Проведено дослідження клінічних штамів *Enterobacteriaceae* з метою визначення їх чутливості до антибіотиків з використанням автоматичної системи і з подальшим генотипуванням детермінант резистентності методом мультиплексною ПЛР. **Результати.** За досліджуваній період серед клінічних штамів *Enterobacteriaceae* було виявлено 10,9 % БЛРС-позитивних ізолятів. Переважно більшість штамів, продуцентів БЛРС – 33,3 % склали *Klebsiella pneumoniae*. Найбільша кількість штамів-продуцентів β-лактамаз розширеного спектру дії було виділено з респіраторного тракту і склало 83,3 %. **Висновки.** Експериментальне дослідження дало нові дані про поширення генетичних детермінант резистентності родини *Enterobacteriaceae*, що продукують БЛРС і їхню роль в розвитку ускладнень. Робота продемонструвала діагностичну цінність молекулярно-біологічних методів для ідентифікації детермінант резистентності у мікроорганізмів.

Ключові слова: Антибіотикорезистентність; β-лактамі антибіотики; ентеробактерії; БЛРС; ПЛР; генотипування

Исследование штаммов *Enterobacteriaceae*, которые продуцируют БЛРС, выделенных из клинических образцов у детей с врождёнными пороками сердца с помощью мультиплексной ПЦР

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Цель. Изучить фенотипы БЛРС и идентификацию избранных генотипов β-лактамаз и частоту их распространения среди клинических штаммов *Enterobacteriaceae*, выделенных у детей с врождёнными пороками сердца. **Методы.** Проведены исследования клинических штаммов *Enterobacteriaceae* с целью определения их чувствительности к антибиотикам с использованием автоматической системы и с последующим генотипированием детерминант резистентности методом мультиплексной ПЦР. **Результаты.** За исследуемый период среди клинических штаммов *Enterobacteriaceae* было выявлено 10,9 % БЛРС-положительных изолятов. Преобладающее большинство штаммов, продуцентов БЛРС – 33,3 % составили *Klebsiella pneumoniae*. Наибольшее количество штаммов-продуцентов β-лактамаз расширенного спектра действия было выделено с респираторного тракта и составило 83,3 %. **Выводы.** Экспериментальное исследование дало новые данные о распространении генетических детерминант резистентности в семействе *Enterobacteriaceae*, которые продуцируют БЛРС и их места в развитии осложнений. Работа продемонстрировала диагностическую ценность молекулярно-биологических методов для идентификации детерминант резистентности у микроорганизмов.

Ключевые слова: Антибиотикорезистентность, β-лактамы антибиотиков, энтеробактерии, БЛРС, ПЦР, генотипирование.

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