

ANTIBIOTIC RESISTANCE IN THE INVASIVE BACTERIA *ESCHERICHIA COLI*

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SUMMARY

Objectives: The beta-lactamases with extended spectrum of activity (ESBL) are medically one of the most important group of enzymes. Another group of beta-lactamases representing of *Enterobacteriaceae* is group of the AmpC-type cephalosporinases. The presented study provides identification and determination of the spectrum of resistance against different and clinically used antimicrobial drugs in the clinical isolates of *Escherichia coli*.

Methods: These isolates had origin in different departments of the L. Pasteur University Hospital in Košice. The goal was the detection of beta-lactamase production with extended-spectrum effect and testing of AmpC-type cephalosporinases by several phenotypic tests in clinical isolates. MALDI-TOF MS analysis was performed on a Microflex MALDI Biotyper. Samples were positively tested for ESBL with the use of the disc diffusion method. PCR were performed with a series of primers designed for the detection of Ambler class A, B and C beta-lactamase genes.

Results: For all 485 isolates, we determined the production of ESBL, which we detected in 166 *E. coli* isolates, which represents a 34.2% prevalence of ESBL production. It is clear from the results that the prevalence of ESBL-producing *E. coli* out of the total number of *E. coli* investigated reached 34.2%. In the monitored period, we confirmed at least one resistance gene from 485 *E. coli* in 188 positive isolates.

Conclusions: We describe a complex ESBL epidemiology. The study revealed a high rate of ESBL-producing *E. coli* isolates; *bla*_{TEM} and *bla*_{SHV} enzymes dominated in ESBL-positive *E. coli* isolates in the L. Pasteur University Hospital in Košice.

Key words: *E. coli*, resistance, ESBL, β -lactamase, AmpC, prevalence

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INTRODUCTION

With the discovery of antibiotics, the healthcare community thought that the battle with infectious diseases was won. However, now that so many bacteria have become resistant to multiple antimicrobial agents, the war has seemingly escalated in favour of the bacteria. Infectious diseases are currently a significant cause of morbidity and mortality worldwide.

From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic “attack”: mutations in gene(s) often associated with the mechanism of action of the compound and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (1).

The family *Enterobacteriaceae* is important for humans as a representation of facultative and obligatory pathogens such as *Escherichia coli*, *Salmonella enterica* or *Yersinia pestis* (2).

The prevalence of extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* is increasing globally, and community-onset infections with ESBL-producing *E. coli* are a major clinical concern in many countries (3–5). Many genera of gram-negative bacteria possess a naturally occurring, chromosomally mediated β -lactamase. These enzymes are thought to have evolved from penicillin-binding proteins, with which they show some

sequence homology. This development was likely due to the selective pressure exerted by β -lactam-producing soil organisms found in the environment (6). The most common cause of resistance to extended spectrum in *E. coli* is the production of ESBLs (7). In the past decade, CTX-M-type ESBLs have replaced TEM- and SHV-type ESBLs in Europe, Canada, and Asia as the most common ESBL type in this species (8). *E. coli* continue to be the most important organisms associated with ESBL-mediated resistance. ESBLs have been classified into types, based on their deduced amino-acid sequences (i.e., TEM, SHV, and CTX-M, PER, VEB, GES, TLA, BES, and OXA types). TEM or SHV derivatives have been the most prevalent types of ESBL, but the prevalence of the CTX-M type has increased dramatically since 1995 in most parts of the world, including Europe, Asia and South America (9–11).

The use of the term multidrug-resistant *Enterobacteriaceae* varies according to the habits of individual hospitals. In general, however, *Enterobacteriaceae* with the production of broad-spectrum β -lactamases of the ESBL, AmpC, or carbapenemases type is referred to as multiresistant. The most common producers of carbapenemases and metallo- β -lactamases are *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (12).

Resistance to third generation cephalosporins is often observed in combination with resistance to fluoroquinolones and aminoglycosides. This type of combined resistance has also increased in the last five years for both *K. pneumoniae* and *E. coli*. An increasing trend is observed in countries with low levels of resistance as well as in countries with high levels of resistance (13).

The aim of the paper was to identify and determine the spectrum of resistance against a variety of clinically used antimicrobial pharmaceuticals for clinical isolates of the genus *E. coli* observed from different departments of the University L. Pasteur Hospital in Košice, Slovakia; and to detect extended-spectrum beta-lactamase production in tested clinical isolates by multiple phenotypic and genotypic assays.

MATERIALS AND METHODS

During the observed period of five years were isolated samples of bacterial cultures from various biological material (urine, wound swab, throat swab, pressure ulcer, vaginal swab, etc.) from patients hospitalized at various clinical departments and outpatient clinics of the University L. Pasteur Hospital in Košice. We processed the collected biological material at the Departments of Clinical and Medical Microbiology, Faculty of Medicine, Pavol Josef Šafárik University in Košice.

We selected only one strain of a given species from one patient, which we isolated from the relevant material first. Sample culture, isolation and identification of bacterial strains were performed using standard diagnostic procedures.

Bacterial Isolates

All *E. coli* isolates (485) from significant clinical samples were analysed for ESBL production. MALDI-TOF MS analysis was performed on a Microflex MALDI Biotyper (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry Biotyper, Bruker Daltonics GmbH, Germany) according to a standard sample preparation protocol of Bruker Daltonik (14). MALDI-TOF mass spectra were subjected to numerical analysis (BioTyper 3.1 software, Bruker Daltonik). *E. coli* strains were confirmed by standard biochemical methods ENTEROtest 16, ENTERO-Rapid 24 (Lachema, Czech Republic). Differences in proportions among categorical data were calculated by the chi-square test with $p < 0.05$ considered significant.

Preliminary Determination of Antibiotic Susceptibility

Was suspended 3–5 colonies in 2 ml of saline to achieve a 0.5 McFarland scale turbidity. The suspension was inoculated on a plate with Mueller Hinton agar (Oxoid) using a swab. Subsequently, antibiotic discs (Oxoid) were applied to the agar surface using a dispenser.

After 24-hour incubation at 37°C, inhibition zones around the discs were measured using a semi-automated Bacmed system (automatic reader and AST analyser).

The evaluation itself was based on information from the European Committee for Antimicrobial Susceptibility Testing (EUCAST-7).

A modified double disc synergy test (DDST) combined with the Clinical and Laboratory Standards Institute method was performed to determine ESBL production (15).

Sensitivity to ESBL-positive strains was determined by a modified disc diffusion assay containing a disc with cefepime (30 µg) and ceftazidime with clavulanic acid (30 + 10 µg) (16).

PCR Amplification and Sequencing

Genotypic detection of ESBL broad-spectrum beta-lactamases was performed by PCR, followed by analyses of the resulting products. The separated fragments were visualized using the MiniBis documentation system (KRD system) and analysed using the GelCapture 3.0 software system. Analysis of genes encoding ESBL production by *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{DHA}, and *AmpC*_{CTT} was performed in BIOER XPcycler (KRD) thermocyclers in a reaction volume of 25 µl using specific primers individual *bla* genes. The fragment length is for *bla*_{TEM} (516 bp), *bla*_{SHV} (475 bp), *bla*_{CTX-M} (543 bp), *bla*_{DHA} (405 bp), and *AmpC*_{CTT} (462 bp).

Sequencing of TEM, SHV and CTX-M Genes

The PCR *bla*_{SHV}, *bla*_{TEM} and *bla*_{CTX-M} products were purified with the PCR SureClean Plus (Applied Biosystems) and sequenced with a Genetic Analyser 3500 (Life technologies).

Nucleotide sequences, deduced amino acid sequences, and phylogenetic relationships were analysed using software packages (SeqScape v2.7 and MicroSeq v2.2).

Statistical Evaluation

Statistical methods of data processing and evaluation were used for statistical comparison of results. The results were processed into tables and graphs (MS Excel 2016, IBM SPSS statistics 22). Statistically, the likelihood of the occurrence of the ESBL-positive *E. coli* strains was processed and it was calculated according to the percentage of the population possibly exposed. The odds ratio (OR) for the monitored indicator of a relative risk was calculated expressing the flagged symptom occurrence ratio to probabilities in the data file depending on the exposure. Even a 95% confidence interval was calculated for the OR.

RESULTS

For strains isolated from clinical material at the L. Pasteur University Hospital in Košice, we compared antibiotic susceptibility, phenotypic test results and the presence of genes encoding ESBL. Over five years, we obtained 485 strains of *Escherichia coli* isolated from an identical number of patients with invasive infections. The MALDI BioTyper evaluation software identified (2.000–3.000) *E. coli* species based on log (score). Log values (scores) lower than 1.7 indicate unreliable identification at the genus level and were therefore not evaluated as positive results. MALDI-TOF MS has shown a high discriminant potential for strain differentiation within the genus. We prospectively identified 485 consecutive clinical isolates (Table 1).

A total of 151 (31.1%) strains were obtained from surgical clinics, 57 (11.7%) strains from the Department of Anaesthesiology

Table 1. Number of strains isolated from 485 samples of biological material in the period 2016–2020

	Year					Total
	2016	2017	2018	2019	2020	
<i>E. coli</i>	51 (10.5%)	84 (17.3%)	140 (28.9%)	114 (23.5%)	96 (19.8%)	485 (100.0%)

Table 2. Overview of representation of *E. coli* in 485 samples of biological material in the period 2016–2020

Genus	Sampling material					
	Urine	Wound swabs	Cannula swabs	Throat swabs	Sputum	Other materials
<i>E. coli</i>	158 (32.6%)	121 (25.0%)	20 (4.1%)	18 (3.7%)	36 (7.4%)	132 (27.2%)

Table 3. Age categorisation of frequency of *E. coli* isolated from 485 patient samples

	Age of patients (years)			Total
	18–40	41–60	≥ 61	
<i>E. coli</i>	18.8%	27.2%	54.0%	100.0%

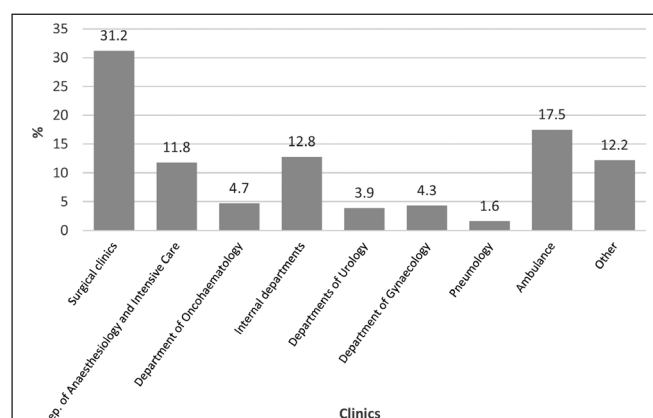
and Intensive Care, 23 (4.7%) strains were from the Department of Onco-Haematology, 62 (12.8%) strains were obtained from internal departments, 85 (17.5%) strains were obtained from the outpatient clinic, and the remaining *E. coli* were obtained from the Departments of Urology 19 (3.9%), Gynaecology 21 (4.3%), Pneumology 8 (1.6%), and from other departments 59 (12.2%) (Fig. 1).

Most *E. coli* were isolated from urine (158, 10.4%), wound swabs (121, 7.9%), sputum (36, 2.7%), cannula swabs (20, 1.3%), throat swabs (18, 1.2%), and other materials (132, 8.7%) (Table 2). The proportion of isolated *E. coli* from patients in relation to age was 18.8% in patients between 18–40 years of age, 27.2% in patients between 41–60 years, and 54.0% in patients more than 61 years old (Table 3).

ESBL Production Results for *E. coli* Strain

The most sensitive method for determining the production of AmpC-type cephalosporinases was the double disc diffusion assay, which detected 16 (3.3%) clinical isolates.

For all 485 isolates, we determined the production of ESBL, which we detected in 166 *E. coli* isolates using a double disc diffusion test (Fig. 2), which represents a 34.2% prevalence of ESBL production.

**Fig. 1.** Frequency of strains obtained from individual departments in the observed period.

The highest proportion of ESBL positive *E. coli* strains came from surgical departments 54 (11.1%) and 31 (6.4%) from internal departments.

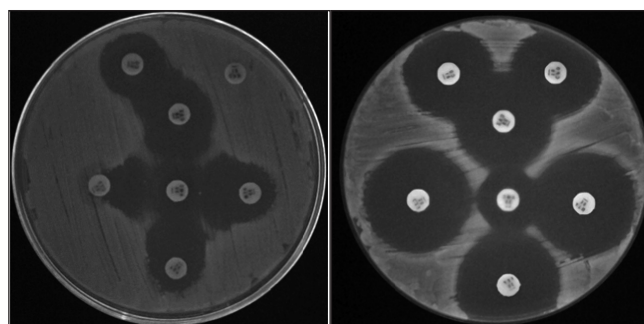
The highest proportion of ESBL-positive *E. coli* was isolated from urine; sputum wound swabs and the lowest from cannula swabs.

When comparing the incidence of ESBL-positive *E. coli* between the sexes, we demonstrated a 1.21-fold (OR = 1.21) higher risk of ESBL-positive strains in the male group compared to the female group. Of the total number of *E. coli* strains isolated, ESBL production was detected in 90 (18.6%) males and in 76 (15.7%) females. Table 4 shows the frequency of ESBL positive *E. coli* by sex in selected wards in the observed period.

It is clear from the results that the prevalence of ESBL-producing *E. coli* out of the total number of *E. coli* investigated reached 34.2%. The risk in patients in the surgical ward is 1.20-fold (OR = 1.20) higher in the age category of 61 years and older than in internal wards with a 95% confidence level.

In the statistical evaluation of OR, the incidence of ESBL-positive *E. coli* strains in the group of men hospitalized in internal wards compared to women was 0.8.

The results showed an increased risk of occurrence ESBL-positive strains depending on gender in surgical departments,

**Fig. 2.** Examination of ESBL production (combination of DDST and CLSI methods).

Left – *Escherichia coli* strain producing ESBL. Right – *Escherichia coli* strain not producing ESBL.

Table 4. Frequency of ESBL positive *E. coli* isolates by sex in selected departments

Sex	Departments	ESBL+	Total number	%
Men	Surgical	41	99	28.5
	Internal	16	27	25.8
Women	Surgical	13	45	9.0
	Internal	15	35	24.2

ESBL – extended-spectrum beta-lactamases

where men had a 74% risk (OR=0.59) compared to a 58% (OR=0.41) risk in women. In the evaluation of individual wards, patients at the Department of Anaesthesiology and Intensive Care have a 2.9-fold (OR=2.86) higher risk of ESBL-positive *E. coli* compared to patients in the surgical wards (OR=0.86).

Statistical analysis confirmed the risk of ESBL-positive strains in urine in women compared to ESBL-positive strains from other materials, demonstrating significance at the 95% level of significance ($p < 0.001$). The risk of ESBL-positive strains in relation to the biological material collected is on the rise in women in whom we found a double risk (OR = 2.489) of ESBL-positive strains in the urine, mainly *E. coli*.

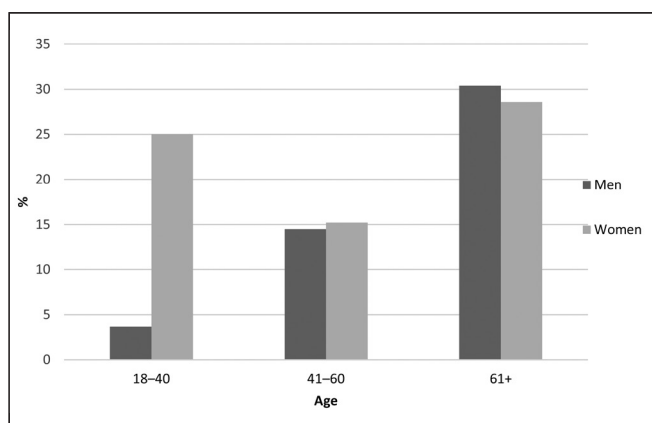
The results showed differences in the incidence of ESBL-positive bacteria not only when comparing sexes, but also when comparing age categories. The level of risk in relation to age and diagnosis indicates its increasing trend with age, more pronounced in the age group 41–60 and ≥ 61 years, where the level of risk of ESBL production in *E. coli* strains is three times higher in men of the age categories compared to the age group of 18–40 years (OR=3.18) (Fig. 3).

As already mentioned, one of the most important risk factors for infection or colonization by ESBL-positive strains is the long-term stay of patients in hospital departments.

Results of Resistance Genes Detection

We identified the genes responsible for resistance to beta-lactam antibiotics by PCR method. In the monitored period, we confirmed at least one resistance gene from 485 *E. coli* in 188 positive isolates. We detected the *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{DHA}, AmpCEBC, AmpCACC, AmpCCIT genes in the investigated bacterial strains.

From the β -lactamases TEM, SHV and CTX-M encoded by the *bla* genes, we detected the *bla*_{TEM} gene in 264 (54.4%) *E. coli*

**Fig. 3.** Frequency of ESBL positive *E. coli* by sex and age of patients.

strains. Of the 485 clinical strains tested, the *bla*_{SHV} genes were present in 164 (33.8%) *E. coli* strains. The *bla*_{CTX-M} gene was detected in 140 (28.9%) *E. coli* (Table 5).

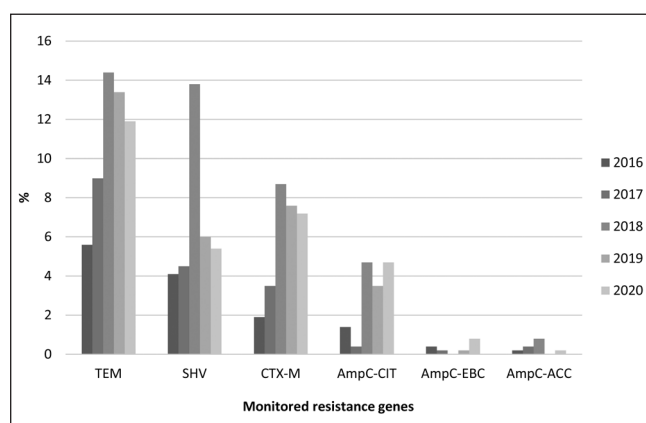
Most isolates carried the *bla*_{CTX-M-15} genes. Sequence analysis of the *bla*_{CTX-M} gene revealed that 85 isolates produce *bla*_{CTX-M-15} enzymes.

When analysing the occurrence of genes encoding beta-lactamase production in *E. coli* isolated from individual sampling materials, it was found that the *bla*_{TEM} gene was most frequently isolated from the urine of 87 (17.9%) patients and in wound swabs from 56 (11.6%) patients, of which resistant strains of *E. coli* were the most frequently isolated (Fig. 4).

In a slightly higher proportion, the *bla*_{SHV} gene was present in strains isolated from the urine of 52 (10.7%) patients and in wound swabs from 40 (8.3%) patients as well as from cannula swabs from 29 (6.0%) patients.

The highest prevalence of the *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes was in strains derived from patients with gastrointestinal and urinary tract diseases. The most isolated resistant strains of *E. coli* came from patients of surgical clinics and in-house clinics, where the majority of patients hospitalized are at increased risk of developing antibiotic resistance due to the necessary antibiotic treatment.

Early identification of patient's colonization by these bacteria during hospitalization is crucial for the early implementation of infection control measures and adequate antimicrobial therapy. Delayed detection of antibiotic-resistant bacteria can lead not only to colonization but also to the subsequent development of infection.

**Fig. 4.** Frequency of resistance genes in *E. coli*.**Table 5.** Frequencies of genes encoding beta-lactamases TEM, SHV and CTX-M from *E. coli* isolated from patient samples

	<i>E. coli</i>
<i>bla</i> _{TEM}	264 (54.5%)
<i>bla</i> _{SHV}	164 (33.8%)
<i>bla</i> _{CTX-M}	140 (28.9%)

DISCUSSION

According to Thakuria and Lahon (17), AmpC-type cephalosporinases confer resistance to bacteria on a wide range of β -lactam antibiotics, including cefoxitin and other extended-spectrum cephalosporins, aztreonam, and combinations of β -lactams with beta-lactamase inhibitors.

The occurrence of plasmid-encoded AmpC β -lactamases has been investigated in various studies (18–20). The prevalence of plasmid-encoded AmpC-producing enterobacteria was relatively low in these studies. This may be due to the identification of AmpC producers by a routine phenotypic screening test in which AmpC producers do not have to be identified (21).

The incidence of ESBL-positive bacteria in the world varies from country to country and hospital to hospital. The most vulnerable group in terms of the possibility of infection or colonization by these resistant bacteria are mainly patients in intensive care units. Long-term hospital stays play an important role in all cases (22).

According to Abdallah et al. (23) the frequent occurrence of resistance to most beta-lactam antibiotics is due to the production of different types of beta-lactamases with an extended spectrum of action that hydrolyse penicillin, first, second, third, fourth, and fifth generation cephalosporins as well as monobactams.

Due to the anatomical differences of the excretory organs – compared to men – women may have urine more contaminated with the surrounding microflora, whether rectal or vaginal. The most common cause of bacterial urinary tract infections is the intestinal bacteria *E. coli* (24).

Lovayová et al. (25) also reported the incidence of infections in a study where the incidence of mainly nosocomial infections caused by *E. coli* was recorded in 83.3% of patients older than 41 years. However, difference in resistance of *E. coli* to selected antibiotics was also recorded among sexes, where higher antibiotic resistance was recorded in women than in men.

The incidence of ESBL-producing enterobacteria is diverse worldwide. A study conducted in Iran found that ESBL produced 41% of enterobacteria strains isolated from urinary tract infections. Of these, 64% were *Klebsiella* spp. and 36% *E. coli* (26). A retrospective cohort study from the USA showed a 19% incidence of ESBL-positive isolates in intra-abdominal infections, with 20% belonging to *E. coli* and 23% to *Klebsiella* spp. (27). ESBL producing *E. coli* is a precariously increasing pathogen. The ESBL prevalence rate is variable in different geographical regions. For instance, in Norway (28), it is lower than 2%, but it can also be very high, over 74%, like in Iran. In Nigeria, ESBL production was found in 21% of isolated strains, with *E. coli* producing ESBL more frequently than *Klebsiella pneumoniae* (25–13%) (29). In a study from Turkey, the rate of ESBL-positive *E. coli* was much higher during 2011 and 2012, 50% and 38%, respectively (30). Similarly, previous studies showed that ESBL-producing *E. coli* was more frequent in patients with several healthcare-associated infections including urinary tract infections, bacteraemia and intestinal tract infections (31, 32).

The above results of authors from different countries and continents point to significant differences in the incidence of ESBL-producing the *Enterobacteriaceae*.

CTX-M type β -lactamases have completely altered the epidemiology of broad-spectrum β -lactamases, as their spread is not limited to medical facilities. It is often spread among outpatients.

Escherichia coli strains, with genes encoding CTX-M production, are often simultaneously resistant to fluoroquinolones. CTX-M-producing strains have many similar multidrug resistance properties to methicillin-resistant (MRSA) strains of golden staphylococci. They occur not only in high-risk patients, but also in patients who have not previously received antibiotics. Their occurrence is no longer limited to medical facilities (33).

A work from Taiwan studying *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter* spp. reports *bla*_{CTX-M}, *bla*_{DHA} and AmpC resistance genes as the predominant (34).

The growing number of nosocomial bacteria resistant to reserve carbapenem antibiotics, imipenem, meropenem or ertapenem, which have so far been effective against multi-resistant gram-negative bacteria, is becoming a serious global problem. This resistance is often accompanied by resistance to other antibiotics. This widens and complicates the picture of multi-resistance of nosocomial bacteria against many other antimicrobial agents that are still effective.

CONCLUSION

Remarkably, as early as the use of antibiotics in the 1940s, the euphoria about the amazing ability of penicillin to treat many infectious diseases was gradually replaced by sobering up with increasing information about treatment failure due to resistance. From the 1940s to the present, the number of antibiotic groups has increased significantly. Nevertheless, bacteria are keeping pace with the development of new antibiotics and may even be a little ahead of the development of new resistance mechanisms.

One of the aims of the work was to analyse the occurrence of some mechanisms of antibiotic resistance in the gram-negative bacterium *Escherichia coli*, which often causes nosocomial infections. We determined the mechanisms of resistance by phenotypic methods and gene detection.

Out of the total number of 485 examined strains, 33 ESBL positive strains with SHV enzyme production, 127 ESBL positive strains with TEM production and 87 ESBL positive strains with CTX-M enzyme production were confirmed. We also confirmed the *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes encoding bacterial beta-lactamase production by PCR analysis by amplification.

The results of the study for the observed period show that the production of AmpC was detected in 188 (12.3%) of *E. coli*.

The most frequently isolated strains were *E. coli* with the production of β -lactamases TEM and CTX-M-15. From the clinical material of patients hospitalized in surgical wards during the observed period, the most frequently identified were strains of *E. coli* with the production of β -lactamases of the AmpC type.

The analysis showed that the most frequently detected type was beta-lactamase TEM, often together with SHV-1, an enzyme with a narrow spectrum of action.

Controlling the incidence and spread of infectious diseases is probably one of the main tasks of modern medicine, as treating an infection caused by a resistant strain of bacteria increases the direct and indirect costs of treatment many times over.

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Conflict of Interests

None declared

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