



THE GENETIC BASIS OF GRAM-NEGATIVE BACTERIA RESISTANT TO ANTIMICROBIALS ISOLATED FROM INVASIVE INFECTIONS IN THE REPUBLIC OF MOLDOVA

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Keywords: antimicrobial resistance, resistance enzymes, resistance genes, gram-negative bacilli, carbapenemases.

Introduction. Despite the efforts made and measures taken to combat antimicrobial resistance, alarming levels of resistance in gram-negative bacteria continue to be reported on a global scale. The antimicrobial resistance mechanisms of these bacteria represent the main cause of therapeutic failures.

Material and methods. A retrospective analysis of strains of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* isolated from patients with invasive infections was conducted for the period 2020-2023. Screening for carbapenemase-producing strains was performed based on sensitivity to antimicrobial agents tested using the Vitek-2 compact automated system. Confirmation of resistance mechanisms was achieved through multiplex PCR molecular biology technique.

Results. The analysis of the obtained data indicates high resistance among strains of *K. pneumoniae* to fluoroquinolones (77.2%), while the majority of *E. coli* strains were resistant to penicillins (53.5%). Concerningly, non-fermentative bacilli strains also present alarming figures, with over 60.0% of *P. aeruginosa* strains resistant to penicillins, cephalosporins, fluoroquinolones, carbapenems, and over 80.0% of *A. baumannii* strains resistant to all tested antimicrobial groups. The resistance enzyme OXA-48 was detected in 91.7% of *K. pneumoniae* strains and 15.6% of *E. coli* strains, while the *bla*NDM resistance gene was detected in 15.9% of *P. aeruginosa* isolates, and the *bla*OXA-23 gene was identified in 55.2% of *A. baumannii* isolates.

Conclusions. The rapid identification of multi-drug resistant gram-negative bacilli ensures the success of therapy for infections caused by them, and monitoring resistance profiles is an essential step for subsequent actions to combat antimicrobial resistance.

Cuvinte-cheie: rezistență la antimicrobiene, enzime de rezistență, gene de rezistență, bacili gram-negativi, carbapenemaze.

BAZELE GENETICE ALE BACTERIILOR GRAM-NEGATIVE REZISTENTE LA ANTIMICROBIENE IZOLATE DIN INFECȚIILE INVAZIVE ÎN REPUBLICA MOLDOVA

Introducere. În pofida eforturilor depuse și măsurilor întreprinse în combaterea rezistenței la antimicrobiene, continuă să fie raportate, în context global, nivele îngrijorătoare de rezistență a bacteriilor gram-negative. Mecanismele de rezistență la antimicrobiene ale acestor bacterii reprezintă principala cauză a eșecurilor terapeutice.

Material și metode. A fost efectuată analiza retrospectivă a tulpinilor de *E. coli*, *K. pneumoniae*, *P. aeruginosa* și *A. baumannii* izolate de la pacienți cu infecții invazive, în perioada 2020-2023. Screeningul tulpinilor producătoare de carbapenemaze s-a efectuat în baza sensibilității la preparatele antimicrobiene testate prin sistemul automat Vitek-2 compact. Confirmarea mecanismelor de rezistență a fost realizată prin tehnica de biologie moleculară – PCR multiplex.

Rezultate. Analiza datelor obținute denotă rezistență înaltă a tulpinilor de *K. pneumoniae* la fluorochinolone (77,2%), în timp ce cele mai multe tulpini de *E. coli* au fost rezistente la peniciline (53,5%). Motive de îngrijorare prezintă și tulpinile de bacili nonfermentativi cu peste 60,0% tulpini de *P. aeruginosa* rezistente la peniciline, cefalosporine, fluorochinolone, carbapeneme și peste 80,0% tulpini de *A. baumannii* rezistente la toate grupele de antimicrobiene testate. Enzima de rezistență OXA-48 a fost detectată la 91,7% tulpini de *K. pneumoniae* și 15,6% tulpini de *E. coli*, în timp ce la 15,9% izolate de *P. aeruginosa* a fost detectată gena de rezistență *bla*NDM, iar la 55,2% izolate de *A. baumannii* s-a identificat gena *bla* OXA-23.

Concluzii. Identificarea rapidă a bacililor gram-negativi multirezistenți asigură succesul terapiei infecțiilor determinate de acestea, iar monitorizarea profilurilor de rezistență este etapa esențială pentru acțiunile ulterioare de combatere a rezistenței la antimicrobiene.

INTRODUCTION

The phenomenon of antimicrobial resistance (AMR) is currently extensively addressed among researchers worldwide due to the rapid spread of multi-drug resistant microorganisms and their impact on public health (1, 2).

Despite all global efforts to reduce the incidence of infections caused by multidrug-resistant strains (MDR), this phenomenon has escalated and seems unstoppable. Furthermore, the spread of antimicrobial resistance is still fuelled by irrational prescription and consumption of antimicrobial agents (1, 2).

Invasive infections are usually associated with high mortality due to delayed appropriate antimicrobial therapy, as well as determining the source of infection.

A major challenge for patients with invasive infections has become multidrug-resistant Gram-negative bacilli (GNB), which represent an important target in AMR surveillance and monitoring (1, 2). In recent decades, *Enterobacteriaceae* and non-fermentative GNB have become the main causative pathogens of invasive infections, accounting for 50–75% of all cases of infections and from 15 to 42% of deaths (3).

Extended-spectrum beta-lactamases (ESBLs) are responsible for hydrolyzing penicillins, monobactams, and third-generation cephalosporins, while carbapenemases and metallo-beta-lactamases (MBLs) can hydrolyze drugs from the carbapenem group (4).

Resistance to carbapenems has been primarily conferred by the production of carbapenemases (5). Currently, 350 variants of carbapenemases have been identified and described worldwide, which are clinically significant, with dominant enzymes including KPC, NDM, VIM, IMP, and OXA-48 (6).

Based on numerous studies conducted worldwide, it has been established that characteristic features of multidrug-resistant strains of non-fermentative Gram-negative bacilli, including *P. aeruginosa*, include the production of enzymes such as NDM, IMP, VIM, and OXA. In strains of *A. baumannii*, the presence of MBL, NDM, and OXA enzymes has been described, but these strains usually also co-harbor various extended-spectrum β -lactamases. The OXA-23 enzyme has been most commonly detected in carbapenem-resistant *A.*

baumannii, and globally, high rates of dissemination (77-100%) of various types of OXA enzymes have been reported in this species (7).

Among multidrug-resistant (MDR) and extensively drug-resistant (XDR) isolates of GNB, ESBL-producing strains and carbapenem-resistant isolates (CRE) have become particularly concerning, especially for medical institutions, due to limited treatment options and high mortality rates (3).

Over 19% of healthcare-associated infections are caused by ESBL-producing bacteria. The mortality rate caused by these bacteria is 57% higher in bloodstream infections compared to mortality from infections caused by sensitive strains (4).

The World Health Organization (WHO) has highlighted the species *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* as a critical priority for the development of new antibiotic options, given that carbapenems, used in the therapy of these infections as a last resort, are no longer effective (1, 2).

For invasive infections, precise diagnosis and initiation of appropriate antimicrobial therapy are essential to increase the patient's chances of survival, thus reducing the high incidence of morbidity and mortality (8).

Molecular-genetic testing of antimicrobial-resistant strains represents a rapid and precise method for detecting the molecular determinants of antimicrobial resistance.

The aim of this study was to determine the resistance profiles and highlight the different variants of carbapenemases detected among *Enterobacteriaceae* and non-fermentative GNB strains isolated from patients with invasive infections in the Republic of Moldova.

MATERIAL AND METHODS

A retrospective cross-sectional study was conducted, including strains of *Enterobacteriaceae* and non-fermentative Gram-negative bacilli resistant to antimicrobial agents, isolated in the 17 laboratories that are part of the national network for epidemiological surveillance of antimicrobial resistance, during the period 2020-2023.

The research included species listed in the global surveillance list of antimicrobial resistance developed by the WHO: *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *A. baumannii*.

The strains were identified using the Vitek2 Compact automated system (BioMerieux), utilizing GN identification cards. Screening of resistant strains was conducted based on their testing with antimicrobial agents, performed using the disc diffusion method, as well as through automated method using the same Vitek2 Compact system, using AST-N204 cards for Enterobacteriaceae, containing antibiotics: amikacin, amoxicillin / acid clavulanic, ampicillin, cefepime, cefotaxime, ceftazidime, ciprofloxacin, ertapenem, fosfomycin, gentamicin, imipenem, meropenem, nitrofurantoin, norfloxacin, piperacillin / tazobactam, and AST-N222 for non-fermentative GNB, containing antibiotics: amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, minocycline, pefloxacin, piperacillin, piperacillin / tazobactam, rifampicin, ticarcillin, ticarcillin / acid clavulanic, tobramycin, which are recommended antimicrobials by the EUCAST standard. Interpretation was also conducted based on the EUCAST standard.

Subsequently, resistant strains were tested using phenotypic methods and molecular biology tests to confirm the presence of resistance mechanisms.

The genetic basis of resistant strains isolated from blood was determined through molecular-genetic testing (multiplex PCR).

A database was created in Excel to systematize the obtained information, followed by the subsequent calculation of respective statistical indices, such as relative values for isolates resistant to antimicrobial agents, relative values for strains positive for phenotypic testing, relative values of resistance enzymes detected in strains investigated through confirmation methods (multiplex PCR), confidence interval for calculated values. These indicators were calculated cumulatively for all four years for each isolated pathogen.

The normality of data distribution was tested using the Shapiro-Wilk and d'Agostino-Pearson methods. Multivariate analysis was conducted to determine variables with significant independent correlations. Z-tests and Chi-square tests were performed to determine the statistical significance of observed differences. Values with $p \leq 0.05$ were considered statistically significant.

The respective research conducted surveillance of GNB isolated from blood, and the chosen criteria and methods were justified because blood is a sterile biosubstrate, making it more likely to represent true infections. Additionally, GNB is the group currently showing the highest resistance rates worldwide.

RESULTS

Throughout the years 2020-2023, a total of 734 strains of Enterobacteriaceae and non-fermentative Gram-negative bacilli isolated from blood were investigated, including 46.4% (95% CI 44.3-48.6) strains of *K. pneumoniae*, 11.8% (95% CI 9.7-14.0) strains of *E. coli*, 33.3% (95% CI 31.2-35.5) strains of *A. baumannii*, and 8.5% (95% CI 6.4-10.7) strains of *P. aeruginosa*.

The analysis of antimicrobial sensitivity of the isolated microorganisms during the period 2020-2023 highlighted high resistance among the studied microorganisms to various groups of antimicrobial agents.

In the analysis of resistance data, it was found that *K. pneumoniae* strains exhibited significantly higher levels of resistance compared to *E. coli* strains. The majority of *K. pneumoniae* isolates were resistant to fluoroquinolones (77.2%; 95% CI 75.1-79.4), followed by cephalosporins and penicillins, with 70.5% (95% CI 68.4-72.7) and 68.7% (95% CI 66.6-70.9) resistant isolates, respectively. *E. coli* showed higher resistance to penicillins – 53.5% (95% CI 51.4-55.7) and fluoroquinolones – 44.9% (95% CI 42.8-47.1).

The analysis of resistance profiles in *E. coli* highlights only 1.7% (95% CI -0.5-3.9) of isolates resistant to carbapenems, while resistance to these agents in *K. pneumoniae* strains was recorded at 40.4% (95% CI 38.3-42.6) of isolates, showing a consistent increase over the years of study.

The combined resistance to cephalosporins, fluoroquinolones, and aminoglycosides in *E. coli* strains isolated during the research period was 8.1% (95% CI 6.0-10.3), while multidrug-resistant *K. pneumoniae* strains constituted 63.4% (95% CI 61.3-65.6).

The results of antimicrobial susceptibility testing for non-fermentative Gram-negative bacilli

(*P. aeruginosa* and *A. baumannii*) isolated during the research period showed similarly alarming levels of resistance. Practically, for all groups of tested antimicrobial agents, these microorganisms exhibited resistance in over 50% of isolates. Furthermore, this resistance showed a consistent increase in these indicators each year.

The highest proportion of resistant strains of *P. aeruginosa* was observed for penicillins, with 65.1% (95% CI 63.0-67.3) of resistant isolates, followed by cephalosporins and fluoroquinolones with a resistance of 63.5% (95% CI 61.4-65.7)

strains. Additionally, a high level of resistance was recorded for carbapenems – 60.3% (95% CI 58.2), and combined resistance to cephalosporins, fluoroquinolones, and aminoglycosides in *P. aeruginosa* strains constituted 52.4% (95% CI 50.3-54.6). Of the total isolates of *A. baumannii*, 99.6% (95% CI 97.5-101.8) were resistant to fluoroquinolones, 93.2% (95% CI 91.1-95.4) to carbapenems, and 88.4% (95% CI 86.3-90.6) to aminoglycosides. The proportion of strains resistant to carbapenems, fluoroquinolones, and aminoglycosides concurrently constituted 80.3% (95% CI 78.2-82.5) (fig. 1).

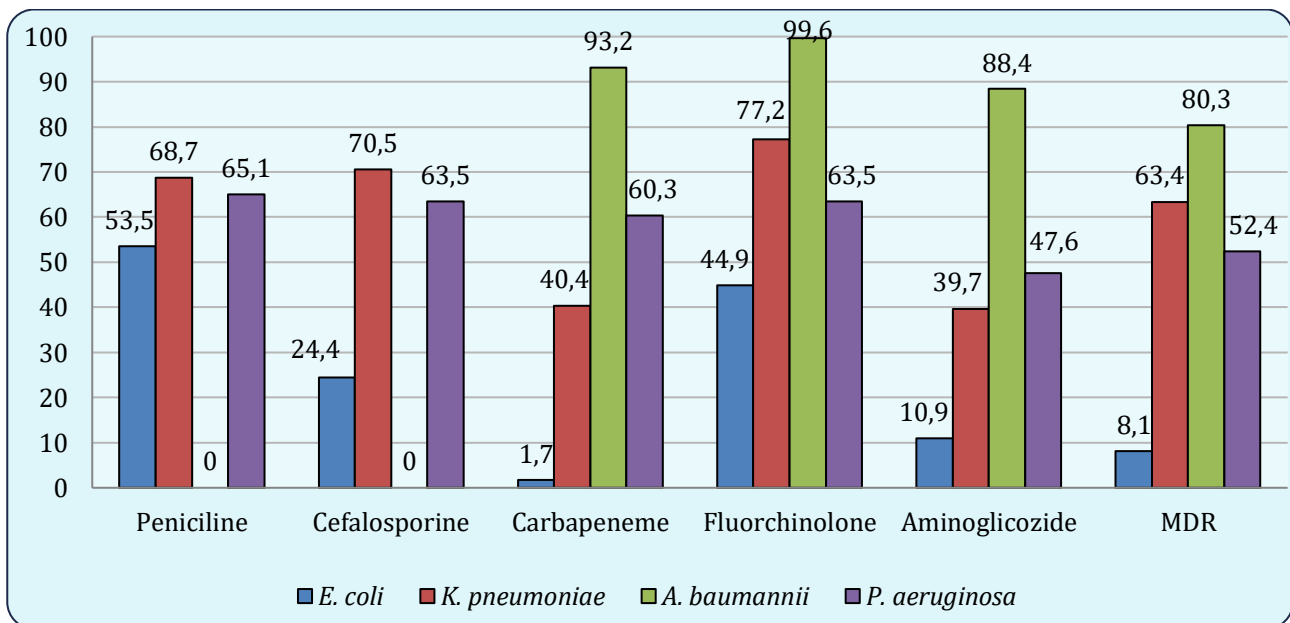


Figure 1. The proportion (%) of *Enterobacteriaceae* and non-fermentative GNB strains resistant to tested antimicrobial groups, 2020-2023.

Based on the antibiotic susceptibility testing, strains suspected to possess resistance mechanisms were selected. The main resistance mechanisms detected in these strains were the production of carbapenemases and extended-spectrum beta-lactamases.

Screening for the production of extended-spectrum beta-lactamases was conducted based on resistance to cephalosporins, and the confirmation of this resistance mechanism was performed only through phenotypic methods (double-disk synergy test, combined disk test). Therefore, this resistance mechanism was not fully elucidated in the respective study.

Screening for the production of carbapenemases by the researched microorganisms was conduc-

ted based on susceptibility to carbapenems (meropenem, imipenem, and ertapenem), and the confirmation of the presence of resistance enzymes was performed through molecular-genetic method – Polymerase Chain Reaction.

Thus, from the isolated strains during the study period, suspected to produce carbapenemases were 54.3% (95% CI 52.2-56.5) of *K. pneumoniae* strains, 96.8% (95% CI 94.5-99.0) of *A. baumannii* strains, 100% (95% CI 97.9-102.2) of *P. aeruginosa* strains, and 1.8% (95% CI -0.4-4.0) of *E. coli* strains.

The PCR test allowed the detection of blaOXA-48, blaKPC, blaVIM, blaIMP, and blaNDM resistance genes in the isolated strains. Thus, the blaOXA-48 gene was present in 91.7% (95% CI 89.6-93.9) of

K. pneumoniae strains, 15.6% (95% CI 13.5-17.8) of *E. coli* strains, and 11.1% (95% CI 9.0-13.3) of *P. aeruginosa* strains. The blaKPC gene was detected in 8.3% (95% CI 6.2-10.5) of *K. pneumoniae* strains and 4.8% (95% CI 2.7-7.0) of *P. aeruginosa* isolates, while the blaNDM gene was recorded in 35.8% (95% CI 33.7-38.0) of *K. pneumoniae* strains, 15.9% (95% CI 13.8-18.1) of *P. aeruginosa* strains, and 1.2% (95% CI -1.0-3.4) of *E. coli* strains. The blaVIM gene was detected only in

P. aeruginosa strains in 12.7% (95% CI 10.6-14.9) of cases. In *A. baumannii* strains, the blaOXA-23 gene was identified in 55.2% (95% CI 53.1-57.4) of isolates, blaOXA-40 in 11.2% (95% CI 9.1-13.4) of isolates, and blaOXA-58 in 20.4% (95% CI 18.3-22.6) of isolates. Additionally, the method identified 2 or even 3 resistance genes simultaneously in the same isolate in 9.0% of cases (95% CI 6.9-11.2) (fig. 2).

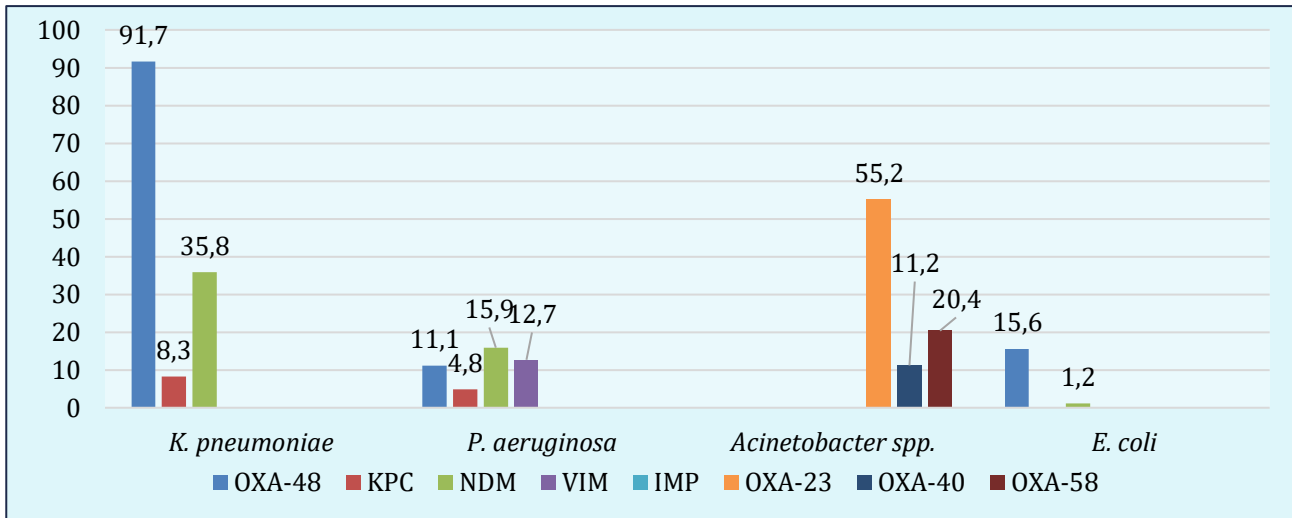


Figure 2. The spectrum of resistance genes detected by PCR method according to the isolated species.

DISCUSSIONS

The study focused on analyzing strains of *Enterobacteriaceae* and non-fermentative gram-negative bacilli resistant as causal agents of invasive infections, and the research results highlighted that *K. pneumoniae* was the main determinant of these infections. Two international studies on invasive infections conducted in two different hospitals in Brazil also found that monomicrobial episodes of invasive infections were largely caused by gram-negative bacteria, selecting those species for research as in the respective work, and also highlighted the prevalence of the *K. pneumoniae* species (9, 10).

Another cross-sectional study conducted in a tertiary hospital in Addis Ababa, Ethiopia found that the majority of bacteria involved in the etiology of invasive infections were gram-negative bacilli (54.2%). Among these microorganisms, *K. pneumoniae* species predominated (32.5%), followed by *Acinetobacter spp.* (20.4%) and *E. coli* (16.5%).

The same consistency in the etiology of invasive infections analyzed was observed in the given

study (4).

Researchers describe *Klebsiella spp.* as the predominant microorganism isolated among gram-negative bacteria in patients with invasive infections from a tertiary care hospital in Malé, Maldives, similar to the research provided. However, in the etiological spectrum of these infections, *E. coli* (8.9%), *Pseudomonas spp.* (12.5%), and *Acinetobacter spp.* (1.5%) follow, which differs from the results obtained in this study (11).

According to the study results, antibiotics from the aminoglycoside group have proven to be the most effective against infections caused by *K. pneumoniae*, which is consistent with the findings of a study conducted at the Laboratory for Research on Hospital Infections (LAPSA FIOCRUZ) in Brazil, where amikacin was the most effective antimicrobial against *K. pneumoniae* (1).

High rates of carbapenem resistance exceeding 90.0% in *K. pneumoniae* may be associated with specific carbapenemase genes that are more com-

monly encountered. Thus, in the same studies conducted in hospitals in Brazil, as mentioned above, the blaKPC resistance gene was most frequently detected in *K. pneumoniae* (1), which does not correspond with the results of this research, where we found that the most commonly detected resistance enzyme in *K. pneumoniae* strains was OXA-48.

Based on prospective surveillance of patients with positive blood cultures at São Rafael Hospital, researchers in Brazil found that the predominant enzymatic variants regarding resistance determinants in multidrug-resistant gram-negative bacilli were SHV, TEM, OXA-1-like, and CTX-M-gp1, while the genes KPC, VIM, OXA-48, NDM, and OXA-23 were characterized as emerging enzymes (8).

In a study conducted on residents of a nursing home in Spain, a significant association was established between the use of medical devices such as venous catheters, urinary catheters, and colonization with carbapenem-resistant GNB. The most commonly isolated pathogen was *K. pneumoniae*, with the blaOXA-48 gene identified. A similar study was conducted in a nursing home in Israel, which also highlighted the prevalence of *K. pneumoniae*. However, the most frequently identified enzyme in these strains was KPC (7).

Similar to the findings in the given study, OXA-48 production has been considered the main mechanism conferring carbapenem resistance among *Enterobacteriaceae* in Tunisia, as well as in Mediterranean countries, which have been considered endemic (12, 13). However, high carbapenem resistance among *A. baumannii* species has typically been associated with the production of OXA-23, OXA-58, and intrinsic carbapenemases such as OXA-51 (12). The same was found in a study conducted by researchers in Brazil, where OXA-23 carbapenemase was the most widespread enzyme detected in *A. baumannii* strains (1, 14, 15).

In contrast to other regions, numerous studies conducted in India have reported isolates of *A. baumannii* responsible for invasive infections, in which the blaNDM gene has been detected (7).

The identification of these resistance enzymes in *A. baumannii* confers resistance to all classes of antimicrobial agents, necessitating the urgent development of new effective antimicrobial agents.

In a study analyzing the mechanisms of resistance in imipenem-resistant *P. aeruginosa* strains responsible for 87 cases of invasive infections in southern Taiwan, Kao et al. found that the most frequently detected carbapenemases were of the VIM type, followed by the OXA type. These findings were consistent with studies conducted in China but differed from those observed in the respective research, where the most frequently detected enzyme in *P. aeruginosa* strains was NDM, followed by VIM (7).

A study conducted by Schäfer et al. at three medical centers in Germany highlighted the prevalence of the blaVIM resistance gene in 30.6% of *P. aeruginosa* strains. Similar data (32% of *P. aeruginosa* strains producing VIM) were recorded by Kateete et al. in a study conducted in Uganda and by Moubareck et al. in research conducted in hospitals in Dubai (7).

An outbreak of extensively drug-resistant *P. aeruginosa* was reported in a tertiary care pediatric hospital in Italy. The study conducted on this outbreak revealed the prevalence of *P. aeruginosa* strains producing the VIM carbapenemase. Furthermore, it was found that the risk of colonization with this species increased with prolonged hospitalization duration (7).

In a study conducted in China, Hu et al. found that all isolates of *E. coli* identified in patients with invasive infections were found to be producers of the NDM enzyme (7). This contradicts the results obtained in the respective research, where the majority of isolated *E. coli* strains were found to carry the OXA-48 enzyme.

Comparable findings to the provided research were obtained in a study conducted in Spain on 121 isolates of carbapenem-resistant *E. coli*, which highlighted the prevalence of the blaOXA-48 gene in 71.9% of strains. The blaVIM gene was detected in 22.3% of strains; the blaKPC gene in 3.3% of strains, the blaNDM gene in 1.7% of strains, and 0.8% of strains harbored the blaIMP gene (16).

A global study highlights carbapenemase NDM as the most commonly identified in isolates of *E. coli*, followed by KPC, whereas studies conducted in Portugal and China highlight the prevalence of the KPC carbapenemase type (16).

CONCLUSIONS

1. Monitoring the resistance profiles of GNB involved in infectious pathology, as well as detecting resistance mechanisms, is indispensable for assessing the spread of AMR and identifying new alternatives in antimicrobial therapy. This process is crucial for implementing national measures to reduce this phenomenon.
2. The results obtained in the study highlight an alarming trend of increasing resistance indices towards most groups of antimicrobial agents. Among these, a high proportion of resistance is noted in *K. pneumoniae* to fluoroquinolones and cephalosporins, in *P. aeruginosa* strains to carbapenems, as well as in *A. baumannii* with extremely high levels of resistance to all tested groups of antimicrobial agents.
3. The investigation of Enterobacteriaceae strains and non-fermentative GNB for the presence of resistance mechanisms has revealed a significant number of strains producing resistance enzymes. Furthermore, in 9.0% of cases, strains with two or more antimicrobial resistance enzymes were recorded.
4. The main recorded resistance mechanisms were the production of carbapenemases, with their types varying from one species to another. Thus, these resistance mechanisms were confirmed in the majority of *A. baumannii* isolates (84.4%), predominantly featuring the OXA-23 carbapenemase, in *K. pneumoniae* and *E. coli* strains - the OXA-48 enzyme, and in *P. aeruginosa* isolates - the NDM metallo-beta-lactamase.
5. The study results, along with the analysis of international studies in this field, point to the need to pay particular attention to GNB, especially regarding the transmission of virulence factors and resistance genes, particularly between species of *K. pneumoniae* and *Acinetobacter* spp.

ETHICAL APPROVAL

Favorable opinion of the Research Ethics Committee of the *Nicolae Testemitanu* State University

of Medicine and Pharmacy, No. 1, dated September 27, 2022.

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