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The Evaluation of Carbapenemase-Producing Strains and the Applicability of Measures to Prevent and Control Their Spread in a Nephrology Hospital

SUMMARY OF THE DOCTORAL THESIS

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Introduction

Antimicrobial resistance is a global public health problem associated with high levels of morbidity and mortality and an increasing incidence, which could be prevented by improving the quality of healthcare services, the access to antimicrobial drugs, as well as through the judicious use of antimicrobial drugs [1–3]. Bacterial infections are common and very diverse, and treatment with antimicrobial drugs is frequently necessary. As a result of selection pressure through inappropriate exposure of microorganisms to antimicrobial substances, the emergence of bacteria exhibiting resistance mechanisms to these substances is favored. Failure to comply with hygiene and spread prevention standards may result in sources of resistant microorganisms that can cause severe, difficult, or impossible to treat infections and can increase the risks associated with medical and surgical procedures [2,4–7].

Beta-lactams are the most widely used antibiotics in medical practice, both for their bactericidal effect on a significant number of bacteria and due to the reduced number of serious side effects compared to other classes. However, exposure of microorganisms to them has resulted in the emergence of resistance mechanisms, especially among Gram-negative bacteria [8,9].

The collection, recording, analysis, and reporting of data obtained from microbiological diagnosis can improve the effort to combat antibiotic resistance. Screening and surveillance for the early detection of resistant microorganisms, both in colonized individuals and in the environment, are useful means for this purpose. Patients hospitalized abroad, those transferred between healthcare facilities, and those immunosuppressed or critically ill can be evaluated for carriage by collecting representative clinical samples and cultivating them on selective culture media, specifically designed for screening. In addition, hygiene measures and measures aimed at combating the irrational consumption of antibiotics, including through training of medical personnel, can help limit the antibiotic resistance phenomenon [7,10].

The problem of antibiotic-resistant microorganisms has also been signaled in Romania. Although there are important efforts to combat this phenomenon, the reported data is still limited. For this reason, efforts to identify, report, and combat the phenomenon must be sustained [11–15].

I. General Section

1. Beta-Lactam Resistance of Gram-Negative Bacteria (GNB)

The multitude of associations between microorganisms and resistance mechanisms is constantly evolving. [1]. Gram-negative bacteria (GNB), both Enterobacterales and non-fermentative species (such as *Pseudomonas* spp. or *Acinetobacter* spp.) can exhibit chromosomal or plasmidic, natural or acquired, constitutive or inducible resistance mechanisms. Mutations in genes encoding target structures or various adaptive mechanisms, such as reducing the number of membrane porins, reducing permeability, developing efflux pumps, etc., can result in antibiotic resistance. Enzymatic mechanisms, however, are among the most important and widespread for these microorganisms, and one of the most important groups is represented by beta-lactamases. Beta-lactamases are enzymes that can inactivate beta-lactams through a hydrolytic mechanism and include penicillinases, extended-spectrum beta-lactamases (ESBLs), cephalosporinases (AmpCs) and carbapenemases. The genes encoding their synthesis are frequently located on mobile genetic elements, such as plasmids and transposons, which allow the spread of resistance both vertically and horizontally, between different strains, species, and even genera. Beta-lactamases are grouped by the Ambler classification into four groups, designated A, B, C, and D, having multiple types and varied action spectra. Carbapenemases in particular are included in classes A (serine-carbapenames, the most common being the KPC and IPM types), B (metallo-beta-lactamases, MBLs, the most common being NDM and VIM) and D (serine-carbapenames, the most common being the OXA-48-like) [1,7,14–19].

Microorganisms that exhibit resistance mechanisms are involved in both severe and difficult to treat infections, but can also be identified in asymptomatic carriers. Colonized individuals, carriers of antibiotic-resistant microorganisms, are at risk of developing infections with these microorganisms under certain unfavorable conditions, as well as of spreading these microorganisms and their resistance mechanisms in hospital and community settings [7,14,15,20–23].

A significant number of beta-lactam and non-beta-lactam antibiotic substances (e.g., various penicillins and inhibitors, various cephalosporins and combinations, carbapenems, aztreonam, vancomycin), as well as metal ions (e.g., zinc ions), are important for the laboratory diagnosis of microbial strains producing beta-lactamases (and implicitly,

carbapenemases). Identification of beta-lactamase-producing microorganisms involves antibiotic susceptibility testing using the standardized Kirby-Bauer disk-diffusion antibiogram and its interpretative reading, determination of minimum inhibitory concentrations through manual and/or automated methods, and detection of resistance genes through molecular methods [7,14,15,20–23].

Additionally, for the specific detection of the presence of various beta-lactamases, techniques that evaluate the in vitro interaction between various antimicrobial substances (e.g., synergy tests) can be used, resistance to certain "key" antibiotics can be assessed, and testing on media with inhibitors can be performed (e.g., Mueller-Hinton with cloxacillin). Furthermore, assays that track the degradation of an antimicrobial substance can be performed, such as tests that evaluate the degradation of carbapenems either colorimetrically, by pH change (e.g. BlueCarba test, Carba NP, etc.), or by other methods, such as the CIM technique (carbapenem inactivation method) and derivatives, detection of carbapenem hydrolysis or direct detection of their presence with MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometry), immunochromatographic techniques (lateral flow assays), etc. [7,14,15,20,24–26].

2. Data on Beta-Lactam Resistance in Romania

As mentioned, an increased level of antimicrobial resistant bacteria has been reported in Romania, including those producing carbapenemases (enzymes with important epidemiological implications); there is progress in combating this phenomenon (guidelines and procedures aimed at training medical personnel, avoiding the primary use of restricted antibiotics, Antibiotic Stewardship programs, screening to detect multidrug resistant microorganisms carriage, etc.), but the currently available data remain insufficient for robust national-level statistical extrapolation [7,11–15,20,27,28]. For these reasons, it is necessary to continue and increase the effort to identify, report, and combat this phenomenon.

II. Personal Contributions

3. Working Hypothesis and General Objectives

Research purpose

Evaluation of beta-lactam-resistant bacterial strains and their resistance mechanisms, identification of measures to prevent and control their spread, and development of accessible phenotypic tests to identify the presence of beta-lactam-resistant microorganisms and their resistance mechanisms.

General objective

Characterization of bacterial strains isolated in a nephrology hospital and of the mechanisms leading to reduced sensitivity to beta-lactams (especially carbapenems), identification of measures to prevent and control their spread, development of a phenotypic test useful in identifying the presence of cephalosporinases and distinguishing the hyper-production of these enzymes from the production of carbapenemases and development of a culture medium for the isolation of carbapenemase-producing enterobacteria, including through carriage and contamination screening.

Specific objectives

1. Characterization of 300-400 bacterial strains (Enterobacterales and non-fermentative Gram-negative bacteria, such as *Pseudomonas* spp. and *Acinetobacter* spp.) isolated over 2 years in the "Dr. Carol Davila" Clinical Hospital of Nephrology, Bucharest, and definition of the mechanisms leading to decreased susceptibility to beta-lactams, with an emphasis on carbapenems, using validated and standardized tests.

2. Development of a culture medium for antibiograms, Mueller-Hinton with oxacillin infusion (resources available in Romania), through which the production of cephalosporinases can be identified (AmpC-type chromosomal cephalosporinases are of major importance), the hyper-production of these enzymes can be differentiated from the production of carbapenemases (situations that may present phenotypically similar on the disk-diffusion antibiogram) and masked ESBLs can be uncovered, by testing 100-150 well-characterized Enterobacterales strains and comparing the results with those obtained on Mueller-Hinton agar and Mueller-Hinton agar with cloxacillin infusion.

3. Development of a recipe for a selective and differential culture medium, using a chromogenic agar base supplemented with molecules with antimicrobial effect (ertapenem,

vancomycin), inhibitory effect on cephalosporinases (oxacillin), and potentiating effect on metallo-beta-lactamases (zinc sulfate), which helps the isolation of carbapenem-resistant microorganisms, especially those resistant through carbapenemase-production, including those from samples collected for carriage and contamination screening.

4. Identification of useful measures for the prevention and control of beta-lactam-resistant microorganisms spread, such as rapid and accessible methods for detecting the presence of these microorganisms in the clinic and laboratory, programs for better interdisciplinary collaboration, judicious prescription and consumption of antimicrobial drugs, and establishment of clear and effective hygiene measures.

5. Writing and publishing scientific papers based on the obtained data, including at least 3 articles (as first author, sole author, or corresponding author), of which at least 1 article in an ISI (Clarivate Analytics) indexed journal with Impact Factor (IF) ≥ 0.5 and 2 articles in journals indexed at least in PubMed.

4. General Research Methodology

The methodology was presented in detail within each chapter in the part related to personal contributions, both in terms of the evaluation of carbapenemase-producing strains from the collaborating nephrology hospital, and the studies through which the cephalosporinase inhibitor potential of oxacillin was demonstrated, and the two culture media were developed.

For the first study, the isolated and characterized bacterial strains, the protocols applied to identify the microorganisms and evaluate the associated resistance mechanisms, the methods used, the statistical tests applied, the software used for graphs and statistical analysis, etc., were described. The strains used in the study were preserved by freezing at -80 °C, both for subsequent evaluations and for other studies in which they could be used.

In the study through which the inhibitory potential of oxacillin was confirmed and the culture medium supplemented with oxacillin was developed, the strain collection selected and used was described too. The working protocol, the molecule, and the reference method used to compare and validate the technique and culture medium, as well as how the statistical analysis was performed, were also described.

For the study through which the culture medium for the detection of carbapenemase-producing microorganisms was developed, which could be used for clinical samples and environmental samples too, the working methodology, the recipe of the developed culture medium, and the tests applied for statistical evaluation were described.

None of the conducted studies involved human subjects, clinical samples, or experimental animals, but only isolated bacterial strains. All data were pseudonymised and collected in accordance with the decisions of the European Parliament and of the Council for the epidemiological surveillance and control of communicable diseases in the European Community (Regulation (EU) 2022/2370 and Regulation (EU) 2022/2371). The ethics committee's approval was requested and obtained from the Scientific Research Ethical Board of the Carol Davila University of Medicine and Pharmacy, Bucharest (Annex 4).

References were managed using Zotero v.9.0.2

5. Characterization of Bacterial Strains Isolated in a Nephrology Hospital and the Mechanisms Leading to Decreased Sensitivity to Beta-Lactams

In the context of antibiotic resistance, the identification and characterization of multidrug-resistant microorganisms are essential. As mentioned, an increased level of antibiotic-resistant microorganisms has also been reported in Romania, but the data available at the national level is still limited. This study aimed to evaluate beta-lactamase-producing Gram-negative bacterial (GNB) strains isolated over two years (2022-2024) in a nephrology hospital in Romania.

A total of 397 Gram-negative bacterial strains isolated between January 2022 and May 2024 that presented positive criteria for antibiotic resistance were evaluated. After isolation on chromogenic media and identification using MALDI-TOF MS, extended standardized disk-diffusion antibiograms were performed, read, and interpreted, following the EUCAST and CA-SFM guidelines, as well as other guidelines proposed and used by the French National Reference Center for Antibiotic Resistance. Testing was completed with evaluation on Mueller-Hinton agar media supplemented with oxacillin or cloxacillin. Enterobacterales strains were not evaluated for resistance to aztreonam-avibactam, and minimum inhibitory concentrations (MICs) for colistin and ceftazidime-avibactam were not determined. For non-fermentative GNB, the MIC for colistin was determined. Standardized phenotypic tests were performed for all strains to detect carbapenemase production: BlueCarba Test (BCT), CIM, mCIM, zCIM, and rCIM. A set of Enterobacterales, all strains of *Pseudomonas* spp., and all strains of *Acinetobacter* baumannii in the study were also evaluated for carbapenemase production by immunochromatographic assays. Statistical evaluation was performed using Microsoft Office Excel v.16.0 and Excel Data Analysis ToolPak. Descriptive statistics were performed for data synthesis, and Cohen's kappa inter-rater agreement and percentage agreement for a specific case were calculated to compare the results of the tests evaluating carbapenemase production.

The 397 strains evaluated included 335 (84.38%) Enterobacterales and 62 (15.62%) non-fermentative GNB. The strains showed significant levels of resistance to the tested antibiotics. Of these, 188 (47.35%) were *Klebsiella pneumoniae* strains; 139/188 (73.93%) showed carbapenem-resistance and carbapenemase-production; 49/188 (26.06%) produced two carbapenemases; and 45/188 (23.93%) showed resistance to all tested antibiotics.

MALDI-TOF identified 28 KPC-producing *K. pneumoniae* strains. Immunochromatographic tests identified NDM, VIM, KPC and OXA-48-like carbapenemases in 48 of the 56 Enterobacterales tested; 12/48 strains produced two carbapenemases. Excluding some inconclusive results determined by BCT, 129/335 Enterobacterales strains were detected as carbapenemase producers by all 6 applied tests (antibiogram, BCT, CIM, zCIM, mCIM, rCIM). Cohen's kappa coefficients indicated very high agreement between the results (0.81 to 0.98). For the results obtained in the case of the 56 strains for which immunochromatographic tests were also performed, high percentage agreements were obtained.

Among the 62 non-fermentative GNB, 33 were *Pseudomonas* spp., and 20 were *Acinetobacter baumannii*; one strain of *Pseudomonas* spp. was sensitive only to colistin and seven only to cefiderocol; four strains of *A. baumannii* were sensitive only to colistin, and three only to cefiderocol. Immunochromatographic assays detected the presence of VIM or IMP-type carbapenemases in 13/33 *Pseudomonas* spp. and OXA-23 and/or OXA-40/-58 in all 20 *A. baumannii* strains. Kappa coefficients showed lower results for the carbapenemase production assays.

False-positive and false-negative results were present, caused both by method limitations and by strains with particular, weak enzymes and/or with increased zinc requirements, associated non-enzymatic resistance mechanisms and particular phenotypes. Highly resistant *K. pneumoniae* strains with a rough culture phenotype have been identified. Susceptibility to some untested antibiotics and molecular evaluation are needed for further data. It should also be noted that the selection process does not allow for adequate incidence and prevalence data reporting.

Bacterial strains with increased resistance to antibiotics, including through the production of two carbapenemases, as well as bacterial strains producing carbapenemases that may go unnoticed by usual screening techniques, but which can associate with difficult-to-treat infections and can disseminate the resistance mechanisms to other bacteria, were identified.

Continuing the characterization of resistant bacterial strains, including through molecular methods, and continuing the collaboration with the hospital where the strains were isolated, while also expanding it to other hospitals in the country, could improve data collection and results with higher statistical relevance may be obtained [15].

6. Development of a Phenotypic Test Useful in Identifying Cephalosporinases and Mechanisms Masked by Cephalosporinases through Inhibition, and Distinguishing Cephalosporinase Hyper-Production from Carbapenemase-Production

The increasing incidence of infections with beta-lactamase-producing GNB represents, as mentioned, a major problem. AmpCs are produced by multiple species of Enterobacterales and can degrade a significant number of beta-lactams, including carbapenems, when associated with membrane alterations. Detection and differentiation of resistance mechanisms are essential for appropriate treatment and for the implementation of measures to prevent and control the spread of resistant strains.

Antibiotic susceptibility testing on Mueller-Hinton agar (MHA) supplemented with cloxacillin (MHC) for AmpC inhibition is a well-known technique for detecting and differentiating AmpC from other beta-lactamases. Cloxacillin is not available in several countries, including Romania. In this study, the potential of oxacillin to be used as an alternative to cloxacillin for AmpC inhibition was evaluated, and Mueller-Hinton supplemented with oxacillin (MHO) as an alternative to MHC for Enterobacterales was developed.

MHO supplemented with various concentrations of oxacillin was compared with MHA and MHC with 250 mg/L cloxacillin. The evaluation was performed using a set of 113 well-characterized beta-lactamase-producing Enterobacterales strains. MHO at a concentration of 300 mg/L to 350 mg/L provided similar results to MHC250. Some strains with particular resistance mechanisms and/or increased sensitivity to antibiotics showed poor growth on inhibitory media, non-specific diameter increases or diameter increases below the detection limit. Oxacillin concentrations for *Pseudomonas* and *Acinetobacter* should be established, as cloxacillin concentrations are different compared to Enterobacterales.

The use of MHO300 proved effective in inhibiting cephalosporinases, allowing the differentiation of AmpC hyper-producing strains from carbapenemases producing ones. The medium allowed the unmasking of hidden mechanisms, such as ESBLs. Oxacillin could also be used in other phenotypic tests to identify resistance mechanisms, similar to cloxacillin, and the culture medium supplemented with oxacillin has the potential to be used as a cheap option for in vitro identification and inhibition of cephalosporinases [14].

7. Development of a Selective and Differential, Chromogenic Culture Medium for the Isolation and Identification of Carbapenemase-Producing Enterobacteria

Carbapenem-resistant carbapenemase-producing microorganisms are a problem not only because of the infections produced and the rapid dissemination, but also due to the potential to colonize exposed individuals. Colonized individuals are at risk of developing infections with these microorganisms and are sources of multidrug-resistant microorganisms. Early carriage detection is important to apply appropriate decontamination and prevention measures. This study aimed to develop a recipe for a chromogenic culture medium supplemented with antibiotics and adjuvants that can allow the isolation of carbapenemase-producing Enterobacterales from representative clinical samples used for screening (such as feces, rectal swabs, etc.), regardless of contamination level, with high sensitivity and specificity.

A chromogenic-based culture medium, supplemented with ertapenem, oxacillin, vancomycin, and zinc sulfate, respecting the pre-established requirements of detection and resistance criteria, was developed. The developed culture medium was inoculated with bacterial suspensions in varying concentrations. A collection of 253 well-characterized Enterobacterales with varied resistance mechanisms, including weakly expressed carbapenemases with reduced hydrolyzing activity, was used.

The culture media allowed the growth of carbapenemase-producing Enterobacterales with high sensitivity (97.4 - 97.6%), even from low concentration suspensions. The strains that gave false-negative results were carbapenemase producers with very low enzymatic activity. The strains that produced false-positive results were non-carbapenemase producers, but with very high ertapenem minimum inhibitory concentrations. These strains are collection strains rarely encountered in clinical practice. Microorganisms resistant to the antimicrobial substances infused in the medium may still grow on the prepared medium.

The developed medium allows the successful isolation of carbapenemase-producing bacteria, including those with low-level resistance. The chromogenic base favors the distinction of genera and species. The medium could be used for the detection of carriage in patients, as well as for the detection of resistant microorganisms in environmental samples [1,5,7,14–16,20,29–34].

Conclusions and Personal Contributions

1. Conclusions

A total of 397 Gram-negative bacterial strains and their resistance mechanisms were evaluated, fulfilling the study objective. High levels of antibiotic resistance and significant variability in carbapenemases and microorganism-carbapenemase associations were identified, with some strains producing two carbapenemases simultaneously.

Interpretive reading of the disk-diffusion antibiogram remains a valuable and reliable method for assessing antibiotic susceptibility and detecting resistance mechanisms. Phenotypic tests for the detection of carbapenemase production show higher sensitivity and specificity in Enterobacterales compared to non-fermentative GNB, but all have limitations. For the proper phenotypic diagnosis of carbapenemase-producing GNB, the evaluation of the antibiogram should be done concurrently with tests that evaluate carbapenemase production. Certain species should be evaluated with a high degree of suspicion in order not to miss strains that produce weak enzymes.

Molecular assessment and additional antibiotic susceptibility testing are needed to gain a complete picture of the evaluated strains. Continuing the collaboration with hospitals to assess resistant strains and to collect demographic data will enable adequate reporting of incidence data, prevalence data, and evolution over time.

The cephalosporinase inhibitory potential of oxacillin as an alternative to cloxacillin has been demonstrated. MHO300 medium is an effective method for detecting AmpC-producing bacteria, differentiating them from those with other resistance mechanisms and unmasking ESBLs. The objective of the research was therefore fulfilled.

The ability to locally produce a culture medium, such as MHO 300 mg/L, could be useful for microbiology laboratories. Establishing the concentration of oxacillin in MHO to detect the presence of AmpC in *Pseudomonas* spp. and *Acinetobacter* spp., and using oxacillin as an alternative to cloxacillin in other assays, are planned.

A selective and differential culture medium with a chromogenic agar base was developed, which allows the successful isolation of carbapenemase-producing bacteria, including those with low-level resistance, with good sensitivity (97.6%), thus fulfilling the objective of the study. This medium could be used in further studies and in practice for the detection and monitoring of patients colonized with carbapenemase-producing microorganisms. Alternatively, the medium could also be used for environmental samples.

Prospects include the development of new media for the isolation of antibiotic-resistant microorganisms and the detection of various phenotypic resistance mechanisms.

Useful methods for the prevention and control of the spread of beta-lactam-resistant microorganisms have been identified and developed, the proposed objective being partially met. Research directions include involvement in the development and implementation of programs aimed at improving interdisciplinary collaboration, antibiotic prescribing and consumption practices, and effective hygiene measures in the hospital environment.

Three articles were published in an ISI (Clarivate Analytics) listed journal with an impact factor (IF) ≥ 0.5 (4.3 - 4.8 at the time of publication), as well as other articles in IDB-listed journals, meeting the publication objective and criteria.

2. Personal contributions

Following an application under the EURO-MEDEX project funded by the Ministry of Research and Digitization, I obtained a grant for the project entitled "Development, optimization and validation of a medium for the isolation of carbapenemase-producing enterobacteria", which I successfully completed.

Results obtained from studies conducted together with the team within the Microbiology II Discipline were exhibited in the form of posters and scientific presentations at conferences and congresses, such as the Carol Davila University of Medicine and Pharmacy Congress, Bucharest 2021, 2022; ECCMID, Lisbon, Portugal 2022; ESCMID Global, Munich, Germany 2026.

Based on the two culture media developed, two patent applications for invention/innovation were registered with OSIM: „Mediu de cultură pentru testarea susceptibilității la antibiotice și identificarea mecanismelor de rezistență la beta-lactamine, procedeu de obținere și utilizare a acestuia”, A/00849/2023, and „Mediu de cultură pentru izolarea enterobacteriilor producătoare de carbapenemaze, procedeu de obținere și utilizare a acestuia”, A/00850/2023; summaries were published in BOPI no. 6/2025, Brevete de invenție (Patents), pos. 138980 A2 and 138981 A2: https://www.osim.ro/images/Publicatii/Inventii/2025/bopi_inv_06_2025.pdf. Additionally, the invention entitled „Produs pentru testarea fenotipică a bacililor Gram negativi producători de carbapenemaze prin inactivarea imipenemului, procedeu de obținere și două metode de utilizare”, A/00399/2026, was registered with OSIM on 19.06.2026.

The 3 main scientific articles were published in the journal Antibiotics (MDPI), Q1, IF 4.8 / 4.3 / 4.6 at the date of publication. Additionally, articles were published in IDB-listed journals.

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1. **Chelaru E-C**, Muntean A-A, Manea I, Hogea M-O, Dinuță C-M, Mazăre M, Muntean M-M, Ghițulescu C-C, Ciomaga B-F, Caracoti C-Ș, Preoteasa D-M, Popa MI. Evaluation of Beta-Lactamase-Producing Strains Isolated in a Tertiary Nephrology Hospital in Romania. Antibiotics. 2026;15(6):580. Q1, IF 4.6/2026, Ch. 5, pg. 55-91. https://doi.org/10.3390/antibiotics15060580.

2. **Chelaru E-C**, Muntean A-A, Muntean M-M, Hogea M-O, Caracoti C-Ș, Ciomaga B-F, Naas T, Popa MI. Oxacillin-Supplemented Mueller-Hinton Agar for In Vitro Inhibition of Ambler Class C β-Lactamases in Enterobacterales. Antibiotics. 2025;14(6):616. Q1, IF 4,3/2025, Ch. 6, pg. 92-116. https://doi.org/10.3390/antibiotics14060616.

3. **Chelaru E-C**, Muntean A-A, Hogea M-O, Muntean M-M, Popa M-I, Popa G-L. The Importance of Carbapenemase-Producing Enterobacterales in African Countries: Evolution and Current Burden. Antibiotics. 2024;13(4):295. Q1, IF 4,8/2024, Ch. 7, pg. 117-132. https://doi.org/10.3390/antibiotics13040295.

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