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SCHOOL OF DOCTORAL STUDIES IN MEDICINE AND PHARMACY

SUMMARY OF THE DOCTORAL THESIS

Title of the thesis: **Infections caused by carbapenemase-producing Enterobacterales members: from microbiological diagnosis to clinical and therapeutic involvements**

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Introduction:

Various types of infections caused by strains belonging to carbapenemase-producing Enterobacterales (CPE) have been spreading worldwide at an alarming rate and are associated with limited therapeutic options, significant morbidity and mortality, which contribute to the reduced success of modern medicine. In addition, recent data suggest the emergence of resistance to colistin, ceftazidime-avibactam, meropenem-vaborbactam, plazomicin.

The management of these infections is a challenge for global health systems and is based on accurate and rapid microbiological diagnosis. Studies have outlined the difficulty of highlighting of different types of carbapenemases, along with the technical challenges of colistin susceptibility testing. According to international guidelines, only the reference broth microdilution method (BMD) is accepted for testing colistin susceptibility, as it is a demanding technique, difficult to implement in routine microbiology laboratory activity and does not allow for the detection of colistin heteroresistance (ChR).

The epidemiology of these isolates is dynamic, the molecular substrates of resistance and virulence markers are constantly evolving, with carbapenemases not being exclusively associated with defined clones.

Doctoral thesis outline:

A) Current state of knowledge:

Presents the main aspects of the subject in relation to the latest literature.

B) Personal contribution:

General purpose: to improve the quality of diagnostic and surveillance methods for CPE infections including colistin-resistant infections in the microbiology laboratory in line with current international methodology.

Specific objectives: to characterise and monitor CPE infections by phenotypic and molecular methods, to describe the clinical and therapeutic characteristics and outcome of patients with CPE isolates, to establish the antibiotic susceptibility profile of CPE strains using standardised methods including BMD for colistin, to demonstrate the possible epidemiological relationship and clonal dissemination of CPE strains.

All the proposed objectives have been achieved by carrying out three studies.

Study No. 1 aimed to assess the molecular epidemiology and clinical impact of CPE strains isolated from hospitalized patients in order to obtain an enlightening picture of the occurrence, spread, clinical, therapeutic and evolutive characteristics. The hypothesis of the study was that there was no difference between the three groups of patients diagnosed with New Delhi metallo- β -lactamase (NDM), *Klebsiella pneumoniae* carbapenemase (KPC) and OXA-48-like.

Study No.2 aimed to perform a comparative evaluation of six phenotypic methods reported to BMD and all major discrepancies (ME) (false resistance results) obtained against this method were further evaluated using the population analysis profile (PAP) method to obtain an improved algorithm for reliable and convenient identification of colistin-resistant CPE isolates in daily practice.



Study No. 3 aimed to perform a detailed phenotypic and genomic characterization of 10 strains of *K. pneumoniae* CPE resistant to colistin (CR) and 2 pairs of ChR strains (parental strains together with corresponding resistant mutants), strains that were collected from two hospitals in Romania between 2017 and 2021, to identify the variety of molecular substrates of CR and ChR, genetic determinants of resistance to other antibiotic classes, confirmation of high-risk circulating clones and dominant plasmids, along with exploration of potential therapeutic options, including new β -lactam/ β -lactamase inhibitor combinations.

General methodology:

The research activity of the present PhD thesis was carried out on a bi-directional basis during the period 01.01.2017 - 13.07.2021.

Study No. 1 was conducted retrospectively in the period 01.01-31.12.2017 in which we included all patients consecutively admitted to the Emergency County Hospital "Dr. Constantin Opreș" Baia Mare who were microbiologically diagnosed with CPE clinical strains from various biological specimens. All non-duplicate CPE clinical isolates were included.

Study No. 2 included 92 non-duplicate CPE clinical strains that were collected from the County Emergency Hospital "Dr. Constantin Opreș" Baia Mare (n=60) from January 2017 to April 2021 (some of the strains were also analysed in study No. 1) and from the County Emergency Hospital Targu Mureș (n=32) from January 2017 to April 2019.

Study No. 3 analysed 10 unique clinical strains of *K. pneumoniae* CPE with CR: 5 selected from patients admitted to the County Emergency Hospital "Dr. Constantin Opreș", Baia Mare, Romania, between January 2017 and April 2021 and 5 from patients in the County Emergency Hospital Târgu Mureș, Romania, between January 2017 and April 2019. In addition, 2 strains of *K. pneumoniae* CPE with ChR obtained from Târgu Mureș in March 2019 and their corresponding mutants with RC were included in the study. This collection of strains was previously phenotypically characterized in study 2.

Results:

Study No.1 outlined *K. pneumoniae* (n=25) as the most commonly diagnosed CPE agent. The representation of NDM, KPC and OXA-48-like carbapenemases was approximately equal. We noted the emergence of two clonal clusters among the 12 positive KPC strains. All CPE isolates showed non-susceptibility to carbapenems, cephalosporins, ciprofloxacin. Respiratory tract infections (n=16) and hospitalization in intensive care unit (ICU) (n=14) ranked highest. The most common comorbidity detected was congestive heart failure (n=11). In most cases (n=15) monotherapy was adopted. Death occurred in 18 patients.

Study No. 2 showed that compared to BMD Micronaut MIC-Strip Colistin, the colistin disc elution method (CBDE) and ChromID Colistin R Agar (COLR) medium showed 100% categorical agreement (CA). Compared to the same reference method, very large discrepancy (VMD) (n=15) was detected for the Etest gradient method, and false resistant results (n=3) was noted only for the Rapid Polymyxin NP Test. The ChR phenomenon in two strains of *K. pneumoniae* CPE was only detected by the PAP method and the Rapid Polymyxin NP Test.

In **study No. 3** whole-genome sequencing (WGS) demonstrated the absence of *mcr* genes, but revealed in 7 strains of *K. pneumoniae* CPE the occurrence of disruptive insertion sequences (IS) (ISKpn25 or ISKpn26) in the *mgrB* gene. Possible mutations responsible for RC and ChR were identified in the *phoP* (L4F), *phoQ* (Q426L, L26Q, L224Q, Q317K), *pmrB* (R256G, P95L, T157P, V352E), and *csrB* (P151S) genes. The isolates belonged to clonal lines ST101 (n = 6), ST 147 (n = 5), ST258 (n = 2) and ST307 (n = 1). All isolates possessed the IncF plasmid. IncL and IncR plasmids were identified in all OXA-48 positive isolates and the IncC plasmid was present in a *bla*_{NDM-1} genome.

Conclusions (general)

Through the complementary application of a diverse range of phenotypic methods, both commercial and reference BMD and PAP, together with molecular techniques: pulsed-field gel electrophoresis (PFGE), polymerase chain reaction (PCR) and WGS, the present work contributes to the optimization of microbiological diagnosis, better characterization of CPE strains, accompanied by a more efficient monitoring of these types of infections in Romania, in accordance with the current methodology used worldwide.



CPE strains had a multidrug-resistant (MDR) profile with limited therapeutic variants, and mortality among patients was high. Monotherapy in urinary tract infections (UTIs) caused by CPE isolates proved to be an appropriate strategy. Ceftazidime-avibactam remains a therapeutic option for *bla*_{KPC-2} and *bla*_{OXA-48} isolates. In some *bla*_{KPC-2} isolates, we found a worrying emergence of resistance to meropenem-vaborbactam in the absence of prior treatment with this new and promising antimicrobial agent. Imipenem/cilastatin-relebactam, tigecycline, gentamicin and trimethoprim-sulfamethoxazole are still potential *in vitro* active agents against some of the pathogens studied.

The current study sheds light on the important challenges related to the phenotypic and genotypic diagnosis of CPE strains co-expressing ChR. Of all the phenotypic techniques used, only the Rapid Polymyxin NP Test and the PAP technique were able to detect ChR in *K. pneumoniae* CPE strains, therefore we suggest the introduction into routine practice of an improved algorithm combining all susceptible results obtained with Vitek 2 Compact, Micronaut MIC-Strip, CBDE and COLR agar with the Rapid Polymyxin NP Test.

The WGS technique has proven to be an innovative and promising strategy that has been able to highlight the multiplicity of resistance determinants to various classes of antibiotics, together with the characterisation of plasmids and circulating clones, thus providing essential data for antimicrobial resistance surveillance. The method revealed on the one hand known mutations T157P, R256G, P95L in *pmrB* or P151S in *crrB*, and on the other hand six previously unreported mutations predicted as intolerant by bioinformatics tools: L4F in *phoP* gene, L224Q, L26Q, Q426L and Q317K in *phoQ* and V352E in *pmrB* which may contribute to CR and ChR. The novel substitution of L224Q in *phoQ* gene was interpreted as having a potentially suppressive effect because in the presence of an IS-modified *mgrB* gene led to low minimum inhibitory concentration (MIC) values for colistin according to BMD.

The PFGE and WGS techniques provided enlightening information on the degree of epidemiological relatedness between strains and their spread in the intrahospital and/or inter-regional environment (Târgu Mureș, Baia Mare). WGS proved silent intrahospital dissemination of ST147 clones with ChR (Târgu Mureș), with close links to another ST147 clone from Baia Mare.

Originality of the thesis:

To the best of our knowledge, the main novelties in the research are as follows:

Study No. 1 is the first research conducted in Romania to investigate significant established risk factors associated with colonisation and/or infection with CPE strains, clinical data, outcome and treatment of CPE positive cases.

Study No. 2 is the first study to comparative evaluate the following combination of phenotypic tests on CPE strains for colistin susceptibility: Vitek 2 Compact, Micronaut MIC-Strip, Etest gradient method on Mueller Hinton E agar (MHE), COLR agar, Rapid Polymyxin NP Test and CBDE versus BMD. First for Romania and, among the few studies in the international literature, using the Rapid Polymyxin NP Test and the PAP technique we were able to diagnose two strains of *K. pneumoniae* CPE with ChR.

In the context of a limited number of such studies in the universal literature, **study 3** is the first Romanian research to focus on the evaluation of molecular determinants of resistance in isolates of *K. pneumoniae* CPE with CR and ChR. For the first time, highlighted the previously unreported mutations predicted as intolerant by bioinformatics tools, L4F in the *phoP* gene, L224Q, L26Q, Q317K and Q426L in *phoQ* gene and V352E in *pmrB* gene, which could contribute to CR and ChR, with the novel substitution L224Q showing a potential suppressive effect in the presence of an IS-altered *mgrB* gene.