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*Article*

# Characterization of Klebsiella pneumoniae Isolates from Croatia Resistant to Cefiderocol

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**Abstract: Background/Objectives:** We conducted this study to evaluate the genotypic and phenotypic profiles of carbapenem resistant *K. pneumoniae* (CRKP)isolates, exhibiting resistance to cefiderocol (FDC) focusing on antibiotic susceptibility, β-lactamase production, genetic environment of *bla*$_{CARB}$ and *bla*$_{ESBL}$genes and molecular epidemiology. FDC is now last line antibiotic for severe infections due to CRKP: **Methods:** Susceptibility to a wide range of antibiotics including carbapenems was determined by disk-diffusion and broth microdilution method. Carbapenemases were screened by modified Hodge test while carbapenem hydrolysis was investigated by CIM and eCIM test. The screening for β-lactamase and fluroquinolone resistance genes was carried out by PCR. Encoding plasmids were characterized by PCR-based replicon typing (PBRT). Inter array-chip test and whole genome sequencing were applied on selected isolates. **Results:** All of the 31 tested isolates exhibited high level resistance to amoxicillin-clavulanate, piperacillin-tazobactam, cefuroxime, expanded-spectrum cephalosporins (ESC), cefepime, ceftolozan-tazobactam and ciprofloxacin and the majority to gentamicin, and amikacin. Colistin preserved activity against 71% and ceftazidime-avibactam against 87% of the isolates. Combined disk method with clavulanic acid was positive in all but one isolate, indicating production of an ESBL. Twenty-eight isolates carried one single carbapenemase encoding gene whereas three harbored double *bla*$_{CARB}$ genes. Among studied isolates 61 % carried *bla*$_{OXA-48}$, 29% *bla*$_{KPC}$ and four *bla*$_{NDM}$ genes... Interarry chip test and WGS identified additional aminoglycoside, sulphonamide and trimethorpim resistance genes. **Conclusion:** To our knowledge, this is the first study on FDC resistance in Croatia. The diffusion of FDR resistant isolates was detected in both hospital and outpatient setting emphasizing the need for "One Health" approach.

**Keywords:** *Klebsiella pneumoniae*; cefiderocol; OXA-48; KPC; metallo-β-lactamase

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## 1. Introduction

Antibiotic resistance is a natural characteristic of microorganisms, existing before the use of antibiotics [1]. The indiscriminate use of antimicrobial in clinical practice, resulted in selective pressure responsible for the spread of antibiotic resistance [2].

Of all opportunistic pathogens *Klebsiella pneumoniae,* alongside *Acinetobacter baumannii,* is the most important due to its capability to cause severe infections like pneumonia in ventilated patients (VAP), bloodstream infections (BSI), urinary tract infections (UTI) and wound infections in immunocompromised and mechanically ventilated patients [3,4]. *K. pneumoniae* isolates of the major concerns are those harbouring extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases (p-Amp-C) and also carbapenemases leading to multidrug-resistant phenotype (MDR) [5–7]. Resistance to carbapenems is caused by enzymatic inactivation mediated by carbapenemases (KPC, IMP, VIM, NDM, OXA-48) spreading by mobile genetic elements, permeability alterations caused by loss of OmpK35 and OmpK36 and hyperexpression of efflux systems [8]. OXA-48 belonging to class D or carbapenem hydrolyzing oxacillinases (CHDL) is now dominant in the majority of European countries [8]. The emergence and spread of MDR strains severely limits therapeutic options, which poses a public health threat. Among carbapenemases, class B or metallo-β-lactamaes (MBLs) represent the greatest challenge to clinicians due to the limited therapeutic options [8]. Colistin is very often the last resort antibiotic, but emergence of colistin resistance in *K. pneumoniae* limits its therapeutic use [9]. Colistin resistance determinants are usually found in ESBL positive and carbapenem resistant *K. pneumoniae* (CRKP*)* resulting in MDR or extensively drug resistance phenotype (XDR) [9]. This poses a challenge to clinicians worldwide who treat these patients and a substantial threat to existing antibiotic armamentarium. The worldwide dissemination of CRKP and its drug resistance transfer poses a global public health [10,11]. New β-lactam-inhibitor combinations such as ceftolozane-tazobactam, ceftazidime-avibactam and imipenem-cilastatin-relebactam are now last resort antibiotics for infections due to CRKP. However, they exert poor activity against isolates producing class B carbapenemases or MBLs [12]. New generation cephalosporins are shown to possess excellent activity against all CRKP [12].

Cefiderocol (FDC) is the first-in-class catechol-siderophore-cephalosporin approved in EU with potent activity against carbapenemase producing Enterobacterales (CPE), *Pseudomonas aeruginosa* (CRPA) and *Acinetobacter baumannii* (CRAB) [13]. The mechanism of action is similar to other cephalosporins, primarily acting with penicillin-binding proteins and with other PBPs to inhibit peptidoglycan cell wall biosynthesis [13]. The catechol functional group of cefiderocol chelates free iron, enabling it to be actively transported into bacterial cell via siderophore iron uptake mechanism and bypass porin channel modification. Its activity is not compromised by upregulation of efflux pumps [13]. It is used for the treatment of complicated urinary-tract infections, hospital acquired bacterial pneumonia and ventilator associated pneumonia [14].

However, there have been increasing reports of correlations between the production of carbapenemases and reduced susceptibility to CFDC [15]. The activity of CFDC can be compromised by metallo-β-lactamases (MBL), of which NDM is the biggest threat [15]. Other recent reports are linking CFDC resistance to the production of other β-lactamases such as extended-spectrum β-lactamases (ESBLs), including PER-1 associated with CFDC resistance in *A. baumannii*, and $bla_{\text{SHV-5}}$ and $bla_{\text{SHV-12}}$ in *K. pneumonia*, respectively. Besides production of β-lactamases, CFDC resistance has been linked to defects in iron transport systems due to mutations of *cir*A and *fiu* genes, impeding drug entry to the bacterial cells [16,17]. The rate of FDC resistance among Enterobacterales in Europe is only 3% (1.5-6%), but is much higher among carbapenem-resistant Enterobacterales (CRE) and reaches 12.5% (7.3-20%) [18]. FCD is being used in Croatia since a few years ago, and resistance to this compound is still rare. The data from 2023 confirmed the rate of 7% (0-16%) [19] among *K. pneumoniae* in general, but there are no studies on the prevalence of resistance among CRKP. Here, we report the emergence and spread of FDC resistance in CRKP producing carbapenemases. We conducted this study to evaluate the genotypic and phenotypic profiles of CRKP isolates, exhibiting

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resistance to FDR, focusing on antibiotic susceptibility, β-lactamase production, genetic environment of *bla*$_{CARB}$ and *bla*$_{ESBL}$genes and molecular epidemiology.

## 2. Results

### *2.1. Isolates and Patients*

The non-copy (one per patient) FDC resistan isolates were recovered from various clinical specimens, including clinically relevant (urine, blood culture, cerebrospinal fluid) or surveillance cultures (throat swab, rectum swab, stool etc) from 31 patients with either *K. pneumoniae* associated infection or colonization. The rate of FDC resistant isolates against total number of CRKP isolates in the participating centers in 2023 was: 31% in UHCS (374/1208), 19% in PH (79/418) and 0% in UHCSM. Data for 2024 are now available for UHCSM which identified 11% (61/552) of resistant isolates and UHCS which detected 10% (22/225).

### *2.2. Antimicrobial Susceptibility and Phenotypic Tests for β-lactamases*

All of the tested isolates exhibited high level resistance to amoxicillin-clavulanate, piperacillin-tazobactam, ceftriaxone, cefepime, ceftolozan-tazobactam and ciprofloxacin and the majority to gentamicin, imipenem, meropenem (97%, n=30), and amikacin (80%, n=25) (Table 1). The resistance to colistin and ceftazidime-avibactam was rarely detected, with 71% (n=22) and 87% (n=27) of the isolates being susceptibile, respectively, as seen in Table 1. Two isolates were allocated to MDR phenotype as they exbitited susceptibility to either carbapenems or aminoglycosides, in addition to colistin and ceftazidime-avibactam. One isolate was PDR since it was resistant to all available antibiotics tested for *K. pneumoniae* in Croatia. MARI indices varied between 0.75 and 1 with mean value of 0.88 and median of 0.87. Combined disk method with clavulanic acid was positive in all but one isolate (97%) while DDST tested positive in 84% (n=26) of the isolates, indicating production of an ESBL. Inhibitor based test with cloxacillin showed uniformly negative result, confirming lack of p-AmpC. Hodge test exhibited higher sensitivity in detecting carbapenemase production with only 2 isolates being false negative (6.4%) compared to CIM which failed to identify carbapenemase in 4 isolates (13%). mCIM was positive in three out of four MBL positive isolates, while one KPC producer demonstrated false positive result.

### *2.3. Molecular Detection of Resistance Genes*

Twenty-eight isolates carried one single carbapenemase encoding gene whereas three harboured double *bla*$_{CARB}$ genes (Table 1). Among studied isolates 61% (n=19) carried *bla*$_{OXA-48}$, 29% (n=9) *bla*$_{KPC}$ and four *bla*$_{NDM}$ genes as shown in Table 1. Double carbapenemases were identified in three isolates (two OXA-48+NDM and one VIM+NDM) (Table 1).

PCR for *bla*$_{CTX-M}$ genes yielded positive result in 26 strains, being phenotypically positive for an ESBL, with all amplicons belonging to cluster 1. *bla*$_{CTX-M-15}$ was the only allelic variant found. *bla*$_{OXA-48}$ *genes* were associated with IS*1999* insertion element upstream of the gene while IS*Ecp* preceded *bla*$_{CTX-M}$ genes. The other β-lactam resistance genes identified were *bla*$_{SHV}$ positive as expected in all isolates and *bla*$_{TEM}$ in 12 isolates. *qnr*B gene was found in one isolate harbouring double carbapenemases.

**Table 1.** Antibiotic susceptibility and beta-lactamase content of FDC-resistant *K. pneumoniae* isolates.

| | Center | Strain | AMC | TZP | CRO | IMI | MEM | GM | AMI | CIP | COL | CZA | C/T | β-lactamase content |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | UHCS | UG65815 | >128 | 128 | >128 | 64 | 128 | 64 | 32 | 64 | 0.5 | R | R | VIM-1, NDM-5, CTX-M |

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| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | SHV |
| 2 | UHCS | UG76341 | >128 | >128 | >128 | 64 | 128 | >128 | >128 | >128 | 0.5 | R | R | OXA-48+NDM, CTX-M, SHV |
| 3 | UHCS | UG72466 | >128 | 128 | >128 | 8 | 32 | 1 | 2 | >128 | 1 | S | R | OXA-48, CTX-M, SHV |
| 4 | UHCS | UG54341 | >128 | 128 | >128 | 1 | 2 | >128 | >128 | >128 | 1 | R | R | NDM, SHV, TEM |
| 5 | UHCS | UG68640 | >128 | 128 | >128 | 64 | >128 | >128 | >128 | >128 | 1 | S | R | OXA-48, CTX-M, SHV |
| 6 | UHCS | UG72747 | >128 | 128 | >128 | 64 | >128 | >128 | >128 | >128 | 64 | S | R | OXA-48, CTX-M, SHV |
| 7 | UHCS | UG78315 | >128 | 128 | >128 | 16 | 32 | 128 | >128 | >128 | 1 | S | R | OXA-48,, CTX-M, SHV |
| 8 | UHCS | UG85877 | >128 | 128 | >128 | 32 | 64 | 128 | >128 | >128 | 0.5 | S | R | OXA-48, CTX-M, SHV |
| 9 | UHCS | UG78871 | >128 | 128 | >128 | 32 | 64 | >128 | >128 | >128 | 128 | S | R | OXA-48, CTX-M, SHV |
| 10 | UHCS | UG81973 | >128 | >128 | >128 | 64 | 64 | >128 | >128 | >128 | 128 | S | R | OXA-48, CTX-M, SHV |
| 11 | UHCS | UG45741 | >128 | 128 | >128 | 16 | 64 | >128 | >128 | >128 | 0.5 | S | R | OXA-48, CTX-M, SHV |
| 12 | UHCS | UG78464 | >128 | 128 | >128 | 32 | 64 | >128 | >128 | >128 | 32 | S | R | OXA-48, CTX-M, SHV |
| 13 | UHCS | UG75475 | >128 | 128 | >128 | 32 | 32 | >128 | >128 | >128 | 2 | S | R | OXA-48, CTX-M, SHV |

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| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14 | UHCSM | VG34989 | >128 | 128 | >128 | 4 | 4 | 64 | 16 | >128 | 16 | S | R | OXA-48, CTX-M, SHV |
| 15 | UHCSM | VG51854 | >128 | 128 | >128 | 32 | 64 | >128 | >128 | >128 | 0.5 | S | R | KPC, TEM, SHV, TEM |
| 16 | UHCSM | VG51612 | >128 | 128 | >128 | 64 | 128 | 64 | 64 | >128 | 0.5 | S | R | KPC, TEM, SHV |
| 17 | UHCSM | VG51788 | >128 | 128 | >128 | 32 | 64 | >128 | >128 | >128 | 0.5 | S | R | KPC, TEM, SHV |
| 18 | UHCSM | VG52055 | >128 | >128 | >128 | 16 | 8 | >128 | >128 | >128 | 0.5 | S | R | KPC, TEM, SHV |
| 19 | UHCSM | VG54301 | >128 | >128 | >128 | 32 | 64 | >128 | >128 | >128 | 8 | S | R | KPC, TEM, SHV, |
| 20 | UHCSM | VG56379 | >128 | >128 | >128 | 64 | >128 | >128 | >128 | >128 | | S | R | KPC, TEM, SHV |
| 21 | PH | 80862-24 | >128 | >128 | >128 | 64 | 128 | >128 | >128 | >128 | 32 | R | R | OXA-48+NDM |
| 22 | PH | 51785-24 | >128 | 128 | 128 | 128 | 128 | 64 | 32 | >128 | 0.5 | S | R | KPC, TEM, SHV, TEM |
| 23 | PH | 46551-24 | >128 | >128 | >128 | 8 | 32 | >128 | >128 | >128 | 0.5 | S | R | OXA-48, CTX-M, SHV |
| 24 | PH | 45896-24 | 128 | 128 | 16 | 16 | 32 | 64 | 32 | >128 | 0.5 | S | R | OXA-48, CTX-M, SHV, TEM |
| 25 | PH | 49359-24 | >128 | >128 | >128 | 8 | 32 | 128 | 32 | >128 | 0.5 | S | R | OXA-48, CTX-M, SHV |
| 26 | PH | 46238-24 | >128 | >128 | >128 | 8 | 32 | >128 | >128 | >128 | 16 | S | R | OXA-48, CTX-M, |

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| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | SHV, TEM |
| 27 | PH | 51750-24 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | 0.5 | S | R | KPC, TEM, SHV |
| 28 | PH | 46092-24 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | 0.5 | S | R | KPC, TEM, SHV |
| 29 | PH | 56620-24 | >128 | >128 | >128 | 8 | 16 | >128 | >128 | >128 | 8 | S | R | OXA-48, CTX-M, SHV |
| 30 | PH | 53807-24 | >128 | >128 | >128 | 8 | 16 | >128 | >128 | >128 | 0.5 | S | R | OXA-48, CTX-M, SHV |
| 31 | PH | 51785-24 | >128 | 128 | >128 | >128 | >128 | >128 | >128 | >128 | 1 | S | R | KPC, TEM, SHV |

Abbreviations: AMC-amoxycillin/clavulanic acid; TZP-piperacillin-tazobactam; CRO-ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; GM-gentamicin; AMI-amikacin; CIP-ciprofloxacin; COL-colistin; C/T-ceftolozane-tazobactam; CZA-ceftazidime-avibactam.

### *2.4. Detection of Resistance Genes by Inter-Array Kit CarbaResist*

Out of four tested representative isolates, two were found positive for *bla*VIM and *bla*NDM genes, respectively. Two isolates were found to carry *bla*OXA-48 gene (Table 2). Combination of two MBL genes was found in one isolate, whereas one harboured combination of OXA-48 and NDM encoding genes as shown in Table 2. Furthermore, aminoglycoside encoding genes *aac(6´)-Ib, aad*A1 and *aad*A2 were identified in three strains. In addition, fluoroquinolone resistance determinant *qnrB* was detected in one isolate being positive for two MBLs. All four isolates tested positive for *sul* 1 gene conferring resistance to sulphonamides with one harbouring *dfrA12* gene, responsible for trimethoprim resistance as well. Finally, genes for efflux pumps were present in three isolates.

**Table 2.** Analysis of four representative *K. pneumoniae* isolates' antibiotic resistance genes by Inter-array chip method.

| Isolate | β-Lactam | Aminoglycosides | Fluoroquinolones | Sulphonamides | Trimethoprim | Efflux Pump |
|---|---|---|---|---|---|---|
| 1 (UG65815) | *bla*VIM *bla*NDM *bla*oxa-1 | *aac(6´)-Ib aadA1 aadA2* | *qnrB* | *Sul*1 | *dfrA12* | |
| 2 (UG76341) | *bla*NDM IS*Ecp-bla*CTX-M-15 *bla*TEM *bla*oxa-1 | *aac(6´)-Ib aphA* | | *Sul*1 | | *oqxA* |
| 3 (VG-34989) | *bla*OXA-48 IS*Ecp-bla*CTX-M-15 *bla*SHV | *aadA2 armA* | | *Sul*1 | | *oqxA oqxB* |
| 4 (8086-24) | *bla*NDM *bla*OXA-48 | *aadA2 rmtC* | | *Sul*1 | | *oqxB* |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | IS*Ecp-bla*CTX-M-15 *bla*SHV | | | | | |

## 2.5. Whole Genome Sequencing

WGS results confirmed the PCR and Inter-array chip results but some disconcordances were identified, particularly, with β-lactam resistance determinants. WGS failed to identify *bla*VIM genes in isolates 1 and 4 as shown in Table 3. However, it detected *bla*NDM genes in isolates 3 and 4 which were missed by Inter-array chip and PCR (Table 3). There were two allelic variants of *bla*NDM genes: *bla*NDM-1 and *bla*NDM-5. Aminoglycoside resistance genes were in concordance using both methods but WGS found additional aph genes in strain 1 not deted by Inter-array chip. Trimethoprim resistance genes were confirmed by both methods, but strain 3 was found to possess *dfrA12* gene not found in chip method. Three different allelic variants of *bla*SHV genes were detected: *bla*SHV-187, *bla*SHV-28, and *bla*SHV-158. Regarding *bla*CTX-M genes, there was only one variant present: *bla*CTX-M-15. The genes encoding efflux pumps (*oqxB)* were confirmed by both molecular methods.

**Table 3.** Whole genome sequencing of four representative isolates. Resistance genes for each antibiotic class are shown and the accession number is provided in the parenthesis.

| Isolate | β-Lactam | Aminoglycosides | Sulphonamide | Trimethoprim | Chloramphenicol | Efflux pumps | Plasmid Inc group |
|---|---|---|---|---|---|---|---|
| 1 (UG65815) | *bla*NDM-5 *bla*OXA-1 *bla*SHV-187 | *Aph(3)-VI* (APPJ01000012) *Aph(3'')Ib* (AF321550) *aadA2* (JQ364967) *aac/6")-Ib* (HQ170510) | *Sul*1 (EU780013) | *dfrA12* (AM040708) | *catB3* (U13880) | | Col(pHAD28) (KU674895 ColpVC (JX133088) IncFIB(K) (JN233704) IncN (AY046276) IncR (DQ449578) IncX3 (JN247852) |
| 2 (UG76341) | *bla*NDM-1 (FN396876) *bla*CTX-M-15 (AY044436) *bla*TEM-1B (AY458016) *bla*OXA-1 | *aac/6")-Ib-cr* (DQ303918) *aac(3")-Ia* (V00359) *aphA* (M28829) | *Sul*1 (EU780013) *Sul*2 (AY034138) | | | *Oqx*B (EU370913) | ColRNAI (DQ298019) IncFIB(K) (JN233704) IncFII(K) (CP000648) IncL (JN626286) |

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| | | | | | | |
|---|---|---|---|---|---|---|
| | (HQ170510 9)<br>*bla*SHV-28 (AF299299) | | | | | |
| 3 (VG-34989) | *bla*NDM-5 (JN104597) *bla*OXA-48 (AY236073 ) *bla*CTX-M-15 (AY044436 ) *bla*SHV-158 (JX121125) | *aadA2* (JQ364967) *armA* (AY220558) | *Sul*1 (U12338) | *dfrA12* (AM040708) | *Oqx*B (EU37091 3) | IncFIB (JN233705) IncL (JN626286) IncX3 (JN247852) |
| 4 (8086-24) | *bla*NDM-5 (FN396876 ) *bla*OXA-48 (AY236073 ) *bla*CTX-M-15 (AY044436 ) *bla*SHV-158 (JX121125) | *aadA2* (D43625) *rmtC* (AB194779) | *Sul*1 (U12338) | | *Oqx*B (EU37091 3) | IncFII (CP000670) IncL (JN626286) |

*2.6. Plasmid Content*

Several plasmid replicons were found including the most frequent IncL associated with all 19 OXA-48 producing organisms while IncX3 was found in three out of four NDM producing organisms. IncN was positive in the strain coharbouring VIM and NDM carbapenemases.

*2.7. MLST*

One of the strains (1, UG 65815) was found to belong to ST20 (*gap*A-2, *pho*E-4, *pgi*-229, *inf*B-3, tonB-4, *rpo*B-4, *mdh*-1) while the other (4, 8086-2-24) was classified as S 4051 (*gap*A-15, *pho*E-1, *pgi*-1, *inf*B-3, *ton*B-31, *rpo*B-1, *mdh*-1). ST for the strain 3 (VG-34989) was retrieved from WGS and was shown to belong to ST15.

## 3. Discussion

Infections caused by MDR bacteria are an alarming problem worldwide although in the last decades a great development of new antibiotics was observed in high-income countries. Access to health care system is associated with an excessive drug uptake, use of biomaterials, and invasive procedures often complicated with nosocomial infections. World Health Organization (WHO) declared antimicrobial resistance as one of the greatest threats to the global health [20]. According to the WHO K. pneumoniae is listed as critical pathogen and a member of a published list of bacteria

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for which new antibiotics are urgently needed [20]. The understanding of molecular mechanisms of antimicrobial resistance is important to cope infections due to these superbugs. Therefore, we aimed to analyse resistance determinants among these critical pathogens.

European studies have shown that FDC is superior to novel-β-lactam inhibitor combinations against CRE (88% vs 66-72%) [12]. In our study ceftazidime-avibactam and colistin exhibited activity against the majority of FDC resistant isolates. The most common species with the problem of FDC resistance worldwide is Enterobacter cloacae complex which could be due to overexpression of chromosomal AmpC cephalosporinase, however, the rate of FDC resistance is constantly increasing among K. pneumoniae [12]. FDC resistance was associated with XDR phenotype in the majority of isolates. Only two isolates demonstrated susceptibility to carbapenems and aminoglycosides, respectively and were categorized as MDR. PDR isolate was resistant to all available antibiotics active against K. pneumoniae, licenced in Croatia.

FDC resistance is usually attributed to multiple resistance mechanisms, including MBL production, ESBL and AmpC positivity, iron-uptake related mutation and ftsl mutation leading to alteration of PBP3, as reported in previous studies [13]. In our study resistance was mostly linked to OXA carbapenemases, although the FDC resistance mechanisms were not analysed in the present study. In contrast to EU studies, our isolates were highly susceptible to ceftazidime-avibactam (87%) and moderately susceptible to colistin (71%, MIC90=128). Ceftolozane-tazobactam did not exert any activity on our FDC resistant isolates. Interestingly, FDC resistant, OXA-48 producing organisms exhibited higher carbapenem MIC values and resistance rates of 100%, compared to previous studies [21,22] in which 18-37% isolates demonstrated resistance to imipenem and 29-47% to meropenem. OXA-enzymes exert weak carbapenem hydrolysis and high level resistance is usually due to other resistance mechanisms such as porin loss or upregulation of efflux pumps. The strain positive only for NDM was susceptible to imipenem and meropenem, and resistant only to ertapenem. In other EU studies FDC resistance was usually identified in NDM producing organisms [23]. Hodge test showed higher sensitivity in detecting carbapenemase activity compared to CIM test which did not identify OXA-48 in some of the strains, contrary to other studies on the sensitivity of phenotypic testing for carbapenemases [24]. False negative tests could be attributed to weak carbapenem hydrolysis exerted by OXA-48. eCIM test was negative in one MBL producing organism. On the other hand, DDST failed to detect ESBLs in KPC producing organisms which were positive in combined disk test with clavulanic acid. This could be explained by inappropriate distance between cephalosporins disks and central disk with clavulanic acid.

The majority of the isolates harboured additional CTX-M β-lactamase. The insertions sequence IS*EcpI* is known to mobilize adjacent sequences, including *bla*$_{\text{CTX-M}}$ genes, by using its own left inverted repeat and increases the expression of the gene [25], which might explain very high cephalosporin MICs among OXA-48 producing organisms, in spite of the fact that this type of CHDL does not hydrolyze cephalosporins. The genetic environment of *bla*$_{\text{OXA-48}}$ genes was consistent with previous work [21].

The study documents dissemination of FDR resistant isolates among K. pneumoniae from participating centers in Croatia. The main finding of the study is that FDC resistance was linked to various carbapenemase types and that the majority of isolates harboured a plethora of other resistance genes as well. This points out to the amazing capacity of K. pneumoniae to acquire resistance determinants to almost all available antibiotics, leaving no therapeutic options left. FDC resistance in other studies was associated mainly with NDM- MBLs, in particular, with ST437, newly emerging clone [26]. Regional differences in the carbapenemase types were observed in this study. In southern region in Split, FDC resistance was most frequently linked to OXA-48, while in Zagreb KPC in most cases accompanied FDC resistance in the hospital setting. On the other hand, in the outpatient setting OXA-48 outnumbered all other carbapenemases. NDM as the sole carbapenemase was recorded in only one case, but double carbapenemaes were reported in three cases, two from Split and one from the outpatient setting. Multiple carbapenemases were already recorded in Croatia during COVID-19 pandemic with OXA-48+NDM as the dominant combination [27], but FDC was

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neither approved for use in Croatia nor tested in routine diagnostic during this period. Fluoroquinolone resistance was attributed to plasmid-mediated *qnr*B gene in one isolate, whereas in other isolates it was probably attributed to mutations of *gyr*A and *par*C genes which is consistent with very high MICs of ciprofloxacin exceeding 128 mg/L.

From the clinical point of view, FDC resistant strain cause difficult to treat infections. Extensive resistance profile has severe clinical implications since it poses challenges to both selection of appropriate empirical and efficient targeted therapy. From a public health perspective, the remarkable ability of *K. pneumoniae* to acquire resistant to newly developed compounds, driving to development of PDR isolates, raises concern about the risk factors, identification of population at risk, and thus measures for the control of their spread, including laboratory identification of PDR isolates are mandatory.

In the present study we aimed to characterize isolates, exhibiting resistance to this last line antibiotic, in order to give insight in their resistome and molecular epidemiology. In this study we combined phenotypic and molecular characterization of resistance traits. This is particularly important in case of carbapenemases, because sometimes they might confer only slight increase in carbapenem MICs as observed with NDM producer, and this is the reason why using molecular approach in addition to phenotypic tests might be helpful.

MBL producing, FDC resistant organisms pose serious therapeutic problem as they are resistant to novel inhibitor combinations such as ceftazidime-avibactam, ceftolozane-tazobactam and imipenem-relebactam. In addition, the presence of arm and rmt genes encoding methylases associated with panaminoglycoside resistance, compromise the use of aminoglycosides. Resistance to FDC was coupled in the majority of cases with ESBL and carbapenemase production, and with resistance to novel β-lactam-inhibitor combinations. The best activity was demonstrated for ceftazidime-avibactam. On the other hand, colistin tested susceptible in approximately 2/3 of the isolates, but the monotherapy is not recommended due to development of heteroresistance and there is also a problem of nephrotoxicity. Aztreonam might exert good activity on MBL positive FDC resistant isolates, but it is not licenced for use in Croatia.

L plasmid, an epidemic plasmid connected with the worldwide dissemination of *bla*$_{\text{OXA-48}}$ genes was detected in our OXA-48 producing organisms, suggesting that it could be responsible for the carbapenem-resistance. IncA/C plasmid was linked to *bla*$_{\text{NDM}}$ genes which is compatible with previous investigations [28] although it is not so unambiguous as with OXA-48 encoding genes as there are also other plasmids such as L/M associated with NDM [29].

STs reported in this study were never identified in Croatia before. In the earlier studies on CRKP the dominant STs were ST29, ST37, ST4871 [22], ST 39, ST437 [27], ST36 and ST258 [28]. ST437 was identified in Italian study in FDC resistant strain carrying *bla*$_{\text{NDM}}$ gene [26]. In Croatian study it harboured *bla*$_{\text{NDM}}$ and *bla*$_{\text{OXA-48}}$ genes [27].

There are several limitations of our study. There was small number of isolates, originating from one country. Moreover, clarification of FDC resistance mechanisms was not done. STs were identified only for three isolates, thus clonal expansion could not be ruled out. On the other hand, the strength of the study is a profound molecular analysis of the isolates, using different methodologies such as Interarray-chip technique and WGS.

## 4. Materials and Methods

### *4.1. Bacterial Isolates and Patients*

This is a descriptive cross section study conducted in two major hospital centers in Croatia: University Hospital Centre Split (UHCS) located in southern Croatia, University Hospital Centre Sestre Milosrdnice (UHCSM) and "Dr. Andrija Štampar Teaching Institute of Public Health" (PH), located in Zagreb. The bacterial isolates included in this study were obtained during routine microbiology testing. The bacterial collection consisted of 31 *K. pneumoniae* isolates with reduced susceptibility to cefiderocol, collected during 2023-2024 in the participating centers. The strains were

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stored at -80° C in the glycerol containing medium, for the purpose of the study and sent to the Clinical Department for Clinical Microbiology and Infection prevention and control of the University Hospital Centre Zagreb (UHCZ) for further analysis. The demographic and clinical data (age, gender, comorbidities and entire hospital courses) were retrospectively analyzed from the internet medical records, in case of hospital isolates. Species identification of the isolates was determined using MALDI-TOF MS (matrix-assisted laser desorption ionization–time of flight mass spectrometry), Biotyper (Bruker, Daltonik GmbH, Bremen, Germany) according to the manufacturer's recommendations.

### *4.2. Antimicrobial Susceptibility Testing (AST) and Phenotypic Tests for Detection of ESBLs, Plasmid-mediated AmpC β-lactamases and Carbapenemases*

The first AST was done by the Kirby-Bauer disk-diffusion test according to the EUCAST guidelines [30] in the participating centers as a part of routine laboratory diagnostic. Isolates exhibiting reduced susceptibility to FDR were subjected to further analysis. Minimum inhibitory concentrations (MICs) were determined by broth dilution method, for research purpose, in Mueller-Hinton broth (Oxoid, Basingstoke, UK) and 96 wells microtiter plates, according to CLSI standards [31] for the following antibiotics: amoxicillin-clavulanate, piperacillin-tazobactam, cefuroxime, expanded-spectrum cephalosporins or ESC (ceftazidime, cefotaxime, ceftriaxone), cefepime, imipenem, meropenem, gentamicin, amikacin, and ciprofloxacin (Sigma Aldrich, USA). MIC results were interpreted following the guidelines outlined in the M100S 110 document [31]. Isolates resistant to at least one carbapenem (imipenem, meropenem and ertapenem) were further tested for colistin susceptibility by broth dilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) [30]. The susceptibility to cefiderocol, ceftazidime-avibactam, sulphametoxazole-trimethoprim, and ceftolozane-tazobactam was determined only by disk-diffusion test. The antibiotic containing disks were provided by Oxoid (Basingstoke, UK). The classification of the resistance phenotype of the strains was as follows: multidrug-resistant (MDR) strains resistant to at least three of the tested antimicrobials belonging to separate antibiotic classes; extensively drug resistant (XDR) strains resistant to all the tested antimicrobials except for two antimicrobial classes, pandrug-resistant (PDR)-strains resistant to all the tested antimicrobial agents [32]. Antibiotics intrinsically resistant in K. pneumoniae such as ampicillin/amoxycillin were excluded. We calculated the multiple antibiotic resistance indices (MARI) as described by Davis and Brown [33] according to the formula: a/b where 'a' was the number of antibiotics an isolate exhibited resistance against the number of antibiotics the isolate was tested against ('b').

ESBL production was screened by double disk-synergy test (DDST) using amoxicillin-clavulanic acid disk opposite to ESC disks [34], and confirmed by CLSI-combined disk test using disks with ESC alone and with addition of clavulanic acid [31]. Augmentation of the inhibition zones of cephalosporin disks of at least 5 mm by clavulanic acid, confirmed ESBL production. *E. coli* ATCC 25922 and *K. pneumoniae* 700603 were used as positive and negative control.

The screening for AmpC production was performed as described previously, considering resistance to cefoxitin as discriminative parameter for the presence of AmpC β-lactamase [35]. Double disk synergy test with a disk supplemented with 500 μg cloxacillin placed between disks containing ceftazidime and cefotaxime on a lawn of the K. pneumoniae isolates was used to confirm p-Amp-C [36]. Distortion of the inhibition zones around ESC disks towards central disk with cloxacillin was considered a positive result [36].

Initial screening for carbapenemase type was conducted in the participating centers for the purpose of routine diagnostic with immunochromatographic OKNV test (OXA-48, KPC, NDM, VIM) [37]. Confirmation of carbapenemase production was done by modified Hodge test (MHT) to confirm the release of carbapenemases by FDR resistant *K. pneumoniae*, according to the CLSI 2017 (M100-S31) [38]. Known carbapenemase positive and negative isolates of *K. pneumoniae* from our collection were used as quality control strains for the MHT. *E. coli* ATCC 25922 strain, susceptible to carbapenems, was cultured overnight, suspended in saline and adjusted to Mc Farland 0.5, and swabbed on MHA.

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Meropenem disks (10µg) were placed in the center of Mueller-Hinton agar MHA plates, and the test isolates were streaked as a thin straight line, from the edge of the disk to the edge of the plate. The plates were incubated in an inverted position at 37 °C overnight. The presence of distorted inhibitory zone (clover-leaf-shape) of *E. coli* ATCC 25922 growth toward the meropenem disk was considered positive result. The isolates proven to possess carbapenemase in MHT were further investigated by EDTA-inhibtor based test. Overnight CRKP culture was spread on the MH agar plate. Imipenem and meropenem disks with and without EDTA were placed on the plate. Cultures were incubated overnight at 37 °C. The augmentation of the inhibition zone around the carbapenem disk for at least 7 mm in the presence of EDTA was considered a positive result [39].

The mCIM and m/eCIM tests were performed according to the Clinical and Laboratory Standards Institute, 2021 guidelines [40] to analyze the carbapenem hydrolysis by the isolates characterized in the study. The mCIM was performed for all the isolates, whereas the eCIM (in conjunction with mCIM) was performed in isolates that initially tested positive for mCIM, as suggested by the CLSI. The interpretation of the test positivity was based on the zone of inhibition of E. coli ATCC 25922 in mCIM and eCIM . For mCIM, 2 ml of overnight Brain-Heart infusion of *K. pneumoniae* isolates was prepared. Further, a 10-µg meropenem disk (Oxoid) was added to the suspension having the test isolate and incubated for 4 hours incubation at 35-37°C. Just before the completion of 4 hr incubation, McFarland 0.5 suspension of E. coli ATCC 25922 was prepared, and lawn cultured (by swabbing) on Mueller-Hinton agar (MHA) plate and left for 3-5 minutes for drying. Subsequently, the meropenem disk was removed from the incubated test tube and placed on the MHA plate and kept in the incubator for 18-24 hours [40]. The following interpretations were considered in the mCIM test: positive (6-15 mm inhibition zone), intermediate (16-18mm-defined as positive if pinpoint colonies are present), and negative (≥ 19 mm inhibition zone [40].

For the eCIM experiment, two tubes (one for mCIM and the other for eCIM) containing 2 ml of TSB were prepared. Twenty µL of 0.5 M EDTA was added into the second tube (for eCIM) and the procedure described previously was repeated with the both tubes [40]. Interpretation of the test positivity was based on the zone of inhibition of E. coli ATCC 25922 on mCIM and eCIM plates. A ≥ 5 mm increase in zone diameter in eCIM experiment as opposed to the respective mCIM plate indicated MBL production. A zone size ≤ 4mm decrease indicated a serine carbapenemase [40]. The strains from own collection, known to be positive for KPC, VIM, NDM and OXA-48 were used as positive and negative controls.

### *4.3. Molecular Detection of Resistance Genes*

An in-house extraction was performed by thermal lysis. Three to five colonies were suspended in ultrapure water and lysed by heating at 95 C for 10 minutes. Cellular debris was removed by centrifugation at 10 000 rpm for 2 minutes. All samples underwent genotypic confirmation of resistance genes by PCR assays. The isolates were screened for the presence of genes encoding broad spectrum and extended-spectrum β-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M,}$) [41–43], and fluoroquinolone resistance genes (*qnr*A, *qnr*B, *qnr*S) [44] using primers and protocols described previously. Plasmid mediated colistin resistance genes *mcr*-1 and *mcr*-2 were sought only in colistin resistant isolates [45]. Multiplex PCR amplification was employed to identify cluster of CTX-M β-lactamase [46], p- AmpC β-lactamase genes [47] and carbapenemase encoding genes of class A, (*bla*$_{KPC}$) class B (*bla*$_{VIM,}$ *bla*$_{IMP}$ and *bla*$_{NDM}$) and carbapenem-hydrolyzing oxacillinases (*bla*$_{OXA-48-like}$) [48]. PCR reactions were carried out in an AC196-Alpha Cycler (PCR max, UK). The presence of insertion sequence preceding the *bla*$_{CTX-M}$ genes was conducted by PCR mapping with forward primer for I*SEcp1* and IS*26* combined MA-3 (reverse for *bla*$_{CTX-M}$ genes) [25]. PCR mapping was applied to analyze the genetic platform surrounding OXA-48 encoding genes, with primers for IS1999 combined with forward and reverse primers for *bla*$_{OXA-48}$ [49]. The positive control strains producing TEM-1, TEM-2 and SHV-1 and SHV-2 were kindly provided by Prof. Adolf Bauernfeind (Max von Pettenkofer Institute, Munich, Germany), CTX-M-15 by Prof. Neil Woodford (Health Protection Agency, London, UK), KPC- 2 by

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Prof. Fred Tenover (Stanford University School of Medicine), and OXA-48 by Dr. Yvonne Pfeifer (Robert Koch Institute, Wernigerode, Germany.

**Table 4.** Primers used in the study. Annealing temperature and the product length is provided.

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| | | | | | |
|---|---|---|---|---|---|
| *bla*TEM | 5'-ATG-AGT-ATT- CAA-CAT-TTC-CG-3′<br>5'-CCA-ATG-CTT-AAT-CAG-TGA-GG-3' | 55 | 850 | 41 | |
| *bla*SHV | 5'-TTC-GCC-TGT-GTA-TTA-TCT-CCC-3<br>5'-TTA-GCG-TTG-CCA-GTG-YTC-GAT-3' | 58 | 1000 | 42 | |
| *bla*CTX-M | 5′-SCS-ATG-TGC-AGY-ACC-AGT-AA-3′<br>5′-CGC-CRA-TAT-GRT-TGG-TGG-TG-3′ | 55 | 550 | 43 | |
| *bla*CTX-M-1 | 5′-AAA-AAT-CAC-TGC-GCC-AGT--TC-3′<br>5′-TTG-GTG-ACG-ATT-TTA-GCC-GC-3′ | 52 | 415 | 46 | |
| *bla*CTX-M-2 | 5′-CGA-CGC-TAC-CCC-TGC-TAT-T--3′<br>5′-CCA-GCG-TCA-GAT-TTT-TCA-GG-3′ | 52 | 552 | 46 | |
| *bla*CTX-M-9 | 5'-CAA-AGA-GAG-TGC-AAC-GGA-TG-3′3′<br>5'ATT-GGA-AAG-CGT-TCA-TCA-CC-3′ | 52 | 205 | 46 | |
| *bla*CTX-M-8 | 5′-TCG-CGT-TAA-GCG-GAT-GAT-GC-3′<br>5′-AAC-CCA-CGA-TGT-GGG-TAG-C | 52 | 666 | 46 | |
| *bla*CTX-M-25 | 5′-GCA-CGA-TGA-CAT-TCG-GG-3′<br>5′-AAC-CCA-CGA-TGT-GGG-TAG-C-3′ | 52 | 327 | 46 | |
| *bla*MOX | 5′GCT-GCT-CAA-GGA-GCA-CAG-GAT-3′′<br>5′CAC-ATT-GAC-ATA-GGT-GTG-GTG-C | 64 | 520 | 47 | |
| *bla*CMY | 5′TGG-CCA-GAA-CTG-ACA-GGC-AAA<br>5′TTT-CTC-CTG-AAC-GTG-GCT-GGT | 64 | 462 | 47 | |
| *bla*DHA | 5′AAC-TTT-CAC-AGG-TGT-GCT-GGG-T<br>CCG-TAC-GCA-TAC-TGG-CTT-TGC | 64 | 405 | 47 | |
| *bla*ACC | 5′AAC-AGC-CTC-AGC-AGC-CGG-TTA<br>TTC-GCC-GCA-ATC-ATC-CCT-AG | 64 | 346 | 47 | |
| *bla*MIR | 5′TCG-GTA-AAG-CCG-ATG-TTG-CGG<br>CTT-CCA-CTG-CGG-CTG-CCA-GTT | 64 | 302 | 47 | |
| *bla*FOX | 5′AAC-ATG-GGG-TAT-CAG-GGA-GAT-G-3′<br>5′CAA-AGC-GCG-TAA-CCG-GAT-TGG-3′ | 64 | 190 | 47 | |
| *bla*IMP | 5′GGAATAGAGTGGCTTAAYTCTC-3′<br>GGTTTAAYAAAACAACCACC-3′ | 52 | 232 | 48 | |
| *bla*VIM | 5-′GATGGTGTTTGGTCGCATA-3′<br>5-′CGAATGCGCAGCACCAG-3′ | 52 | 390 | 48 | |
| *bla*NDM | 5′-GGTTTGGCGATCTGGTTTTC-3′<br>5′-CGGAATGGCTCATCACGATC-3′ | 52 | 621 | 48 | |
| *bla*KPC | 5′CGTCTAGTTCTGCTGTCTTG-3′<br>5′-CTTGTCATCCTTGTTAGGCG-3′ | 52 | 798 | 48 | |
| *bla*OXA-48 | 5′-GCGTGGTTAAGGATGAACAC-3′<br>5′-CATCAAGTTCAACCCAACCG-3′ | 52 | 438 | 48 | |

### *4.4. Interarray-Chip Method*

Four *K. pneumoniae* isolates (one or two from each center) were genotyped by an Inter-array chip according to the manufacturer's recommendations (Inter-array, fzmb GmbH, Bad Langensalza, Germany). The Inter-array genotyping Kit CarbaResist detects broad-spectrum β-lactamases, p-AmpC, ESBLs and carbapenemases and numerous other resistance genes (https://www.inter-

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array.com/further-genotyping-kits). RNA-free, unfragmented genomic DNA was isolated from pure culture of the test strains, amplified and internally labelled with biotin-dUDP according to the linear PCR amplification protocol using the antisense primer of the different targets only. Single-stranded DNA (ssDNA) reaction products were obtained. The biotin-labelled ssDNA was transferred to the ArrayWell and hybridised to DNA oligonucleotide microarrays with 230 probes for different β-lactam, aminoglycoside, fluoroquinolone, sulphonamide, trimethoprim and colistin resistance genes. HRP-conjugated streptavidin was bound to the hybridised biotin-labelled ssDNA stains and visualised by enzymatic reaction. The INTER-VISION Reader was used to evaluate the spots and their intensities automatically on the basis of a digital image of the microarray. The samples obtained from the strains tested in the study were automatically analysed for the presence or absence of specific probes, cross-checked against a database and then information about existing resistances was output.

### *4.5. Whole Genome Sequencing (WGS)*

Four representative isolates were subjected to WGS [50]. First, the strains were cultivated in Tryptic Soy Broth (TSB) and Casein-Peptone Soymeal-Peptone (CASO) Broth (Merck Millipore, MA, USA) at 37 °C overnight. Then, the genomic DNA was extracted using the QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA extracts were sent to the Next Generation Sequencing Facility of the Vienna Biocenter for sequencing using Illumina's NextSeq1000 system according to the manufacturer's instructions. The single reads obtained were assembled and analysed using the webservers and services of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org (accessed on on 13. 01. 2023 [50]. The sequences were deposited in the NCBI Gen Bank, and the accession numbers were provided in the Table 3.

### *4.6. Characterization of Plasmids*

Plasmid DNA of clinical isolates and their transconjugants was extracted with Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). After staining with ethidium bromide, the DNA was visualized by ultraviolet light.

PCR-based replicon typing (PBRT) [51] was used for molecular typing of plasmids conferring resistance in Enterobacterales. Eighteen pairs of primers were used, including five multiplex and three simplex PCR in order to assess the plasmid incompatibility group. Updated method was used for IncL plasmid, which usually carry *bla*$_{\text{OXA-48}}$ genes [52]. Positive control strains for PBRT were obtained from dr. A. Carattoli (Instituto Superiore di Sanita, Rome, Italy).

### *4.7. Genotyping of the Isolates*

MLST was applied on two representative *K. pneumoniae* isolates (1 and 4) by amplifying seven housekeeping genes (*gap*, *pho*, *pgi*, *inf*, *ton*B, *rpo*B, *mdh*) according to the protocol of Diancourt et al [53]. Sequence analysis of PCR amplicons was carried out by Eurofins Genomics (https://eurofingenomics.eu).

## 5. Conclusions

To our knowledge, this is the first study on FDC resistance in Croatia. The diffusion of FDR resistant isolates was detected in both hospital and outpatient setting emphasising the need for one health approach. Croatia is one of the countries with a high rate of antibiotic resistance, and where antibiotics are used excessively and often inappropriately, resulting in a high rate of carbapenem resistance (19%) according to EARS data [18]. The fact that cefiderocol resistance was coupled with carbapenemase and ESBL production and in some cases with colistin resistance, left only a few or no therapeutic options available. The emergence and spread of this dangerous superbug raises concern and call for a change in public health policy regarding the use of antibiotics. New β-lactam antibiotics

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and cefiderocol remain an important addition to the antibiotic armamentarium, but their use must be constantly monitored to avoid the rapid development of resistance. Although they have shown a great promise, experience with their use is still limited.

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**Informed Consent Statement:** Not applicable, this is retrospective *in vitro* study. The study was approved by the Ethical Committee, class: 053-01/23-01/1, number: 251-758-24-31

**Data Availability Statement:** The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

AMC-amoxycillin/clavulanic acid; TZP-piperacillin-tazobactam; CXM-cefuroxime; CAZ-ceftazidime; CTX-cefotaxime; CRO- ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; GM-gentamicin; AMI-amikacin; CIP-ciprofloxacin; COL-colistin, C/T-ceftolozane-tazobactam; CZA-ceftazidime-avibactam;FCD-cefoderpcpč CIM-carbapenem inactivation method; eCIM-EDTA-CIMtest; ESC-expanded-spectrum cephalosporins, MIC-minimum inhibitory concentration, DDST-double disk synergy test; MBL-metallo-β-lactamase; CHDL-carbapenem-hydrolyzing oxacillinase; CRKP-carbapenem-resistant *Klebsiella pneumoniae*

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