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# Carbapenem resistance determinants in *Klebsiella pneumoniae* strains isolated from blood cultures-comparative analysis of molecular and phenotypic methods

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## Abstract

***Introduction:*** *This study provides data on carbapenemases identified in carbapenem-resistant Klebsiella pneumoniae (CR-KP) isolated from blood-cultures by the multiplex molecular method.* ***Material and method****: Between October 2016 and September 2017, 47 non-duplicate Klebsiella pneumoniae (KP) were isolated from blood cultures, from hospitalized patients in the Regional Institute of Gastroenterology and Hepathology, Cluj-Napoca, Romania. Identification and antimicrobial susceptibility tests (AST) were performed by Vitek 2 Compact. The combination disks test (CDT) was used for phenotypic analysis and the LightCycler® Multiplex DNA assay was used to detect and identify the carbapenemases by the LightCycler®z 480 Instrument. The following targets were chosen:* $bla_{KPC}$, $bla_{NDM}$, $bla_{GES}$, $bla_{IMP}$ *and* $bla_{OXA-48}$ *genes and the Cobas® 4800 software variant 2.2.0 was used for the results interpretation.* ***Results****: Taking into consideration the meropenem minimum inhibitory concentration (MIC), 29 KP were susceptible and 18 were not-susceptible (MIC≥0.5 μg ml-1). In the CR-KP group, the CDT identified OXA-48 (10/18) and KPC (7/18) producers. One isolate showed a noninterpretable profile. The multiplex molecular analyses confirmed the carbapenemases production as: 9 CR-KP were KPC and OXA-48 co-producers, 8 were OXA-48 and one was KPC producing strains. In CR-KP group, we found a significant correlation between the CDT and RT-PCR tests results, concerning KPC (p = 0.671). Eight phenotypic results were confirmed by molecular LightCycler® Multiplex DNA assay. For CR-KP co-producers (KPC and OXA-48), the CDT could indicate only one carbapenem-hydrolyzing enzyme.* ***Conclusion****: This study highlights the CR-KP co-producers (OXA-48 and KPC). OXA-48-like is more frequently encountered in our area than other carbapenemases.*

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## Introduction

Surveillance of antimicrobial resistance (AMR) including CR is important worldwide. Several projects were developed in the last few years (e.g. EARS-NET-European Center for Diseases Prevention and Control, CAESAR- Eastern European Surveillance of AMR), but in many countries AMR surveillance is not yet initiated or is under-resourced (1).

The severity of systemic infections with carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) is important due to its high mortality rate as well as the increased hospitalization costs.

From a structural point of view ß-lactamases are classified in four molecular classes. Serine enzymes, molecular classes A, C, and D, have serine in their active region. Metalloenzymes belong to the molecular B class (MBL). Considering their amino acid identity, carbapenemases are part of classes A, B and D (2–4). Class A members hydrolyze carbapenems, cephalosporins, penicillins, aztreonam. Their hydrolytic activity is inhibited by clavulanic acid and tazobactam. The $bla_{KPC}$ gene is frequently detected in KP and hydrolysis all β-lactams with specific hydrolysis of broad spectrum cephalosporins (eg. cefotaxime type). The KPC gene (*Klebsiella pneumoniae* carbapenemase) was first detected in 1996 in the USA. The $bla_{GES}$ gene (Guiana extended spectrum) was isolated first in 2000 from a KP strain and encodes a broad-spectrum hydrolysis enzyme (penicillins and cephalosporins), but with a weak action on imipenem (2,3). OXA (oxacillin-hydrolyzing) is a carbapenemase belonging to class D β-lactamases. The $bla_{OXA-48}$ gene is transferred by plasmids, provides resistance to oxacillin and other anti-staphylococcal penicillins and was detected in KP. Its structure is 50% different from the rest of the OXA-48-like group (2).

Bacterial resistance is common in Gram-negative bacilli (GNB). Recently, Muntean et all published a statistically significant increasing trend regarding to CR-GNB observed particularly in the intensive care unit of a tertiary healthcare unit in the southwestern part of Romania, with the intention of improving prevention and control of nosocomial infection policies (5).

Another study analyzed the *Proteeae* strains within the difficult-to-treat (DTR) phenotype and identified the factors that influence the infection with these bacteria and the predictive factors for the patient's evolution. Musuroi et all concluded in their study that the DTR phenotype has been associated with the species *Providencia stuartii*, with invasive exogenous factors and with an increased fatality (6).

The Golden standard test for molecular carbapenemases detection is the polymerase chain reaction (PCR). To date, there is a limited number of carbapenemase-primers available. Novel carbapenemases may remain undetected.

A new multiplex fluorescence-based RT-PCR which runs on microfluidic Revogene platform was developed and is designed for the detection of genes encoding five carbapenemases: NDM, VIM, IMP, KPC and OXA-48 from *Enterobacterales*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The investigators reported a 100% sensitivity and specificity and characterized this assay as suitable for microbiology laboratories (4).

The objectives of our study were: i) to identify the carbapenemase type using a multiplex RT-PCR with fluorescent labeled hydrolysis probes; to our knowledge this technique is used for the

first time in Romania; ii) to test agreement between the types of carbapenemase identified by phenotypic methods and the detected carbapenemase genes.

## Material and methods

Between October 2016 and September 2017, 47 non-duplicate KP were isolated from blood cultures, from hospitalized patients with sepsis in the Regional Institute of Gastroenterology and Hepathology, Cluj-Napoca, Romania, a University Hospital with 420 beds.

This retrospective study was approved by the ethics committee of the Regional Institute of Gastroenterology and Hepathology, Cluj-Napoca, Romania (Nr. 2082/16.02.2017) and by the ethics committee of the Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania (Nr.478/21.11.2019). The need for informed consent was waived because the study used only isolated pathogens and individual information was anonymized.

The collected blood samples were incubated in the BactAlert3D System (bioMérieux, Inc., Durham, NC), according to the work protocol recommended by the manufacturer.

The KP pure culture was obtained after overnight incubation at 35°C on Columbia sheep blood agar plates (bioMérieux SA, Marcy-l'Etoile, France).

Isolates identification (ID) and antibiotic susceptibility testing (AST) were performed with the Vitek 2 Compact System (bioMérieux, Inc., Durham, NC) with the ID/AST cards. The reference strains used were: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 1705, *Klebsiella pneumoniae* ATCC 1706, *Klebsiella pneumoniae* ATCC 700603. We tested the susceptibility of KP isolates for different antibiotic families: ß-Lactams (three carbapenems included), aminoglycosides, quinolones, trimethoprim-sulfamethoxazole. and colistin (7).

The Advanced Expert System showed CR-KP strains based on MIC values for imipenem, meropenem and ertapenem, interpreted according to EUCAST guidelines 2017 (8). In order to detect carbapenemase producers, we used as inclusion criteria the EUCAST recommended screening cut-off value for meropenem (MIC >0.12 mg/L) (9). The modified carbapenem inactivation method (mCIM) was used to detect the carbapenemase-producers (7) following the Clinical and Laboratory Standards Institute (CLSI) guidelines instructions (10). The combination disks test (KPC, MBL, OXA-48 Confirm kit, Rosco Diagnostica A/S, Taastrup, Denmark) was performed for phenotypic confirmation of the carbapenemase type, in accordance with EUCAST guidelines (9).

The High Pure PCR Template Preparation kit version 21 (Roche Diagnostics GmbH, Mannheim, Germany) was used to manually obtain the genetic material from bacterial cells (suspension of $10^6$-$10^9$) lysed with lysozyme treatment (MP Biomedicals, LLC, BP50067 lllkirch, France), according to the microbial culture extraction protocol. The followed steps were: sample lysis, microbial DNA binding to the glass fibers prepacked in the High Pure Filter Tube, washing and elution (11). Eluted DNA had high purity, free of RNA, other cellular components and DNA polymerase inhibitors.

For the first time in Romania, the LightMix® modular carbapenemase kits (TIB MOLBIOL Syntheselabor GmbH | Eresburgstr. 22-23 | D-12103 Berlin | Germany) designated for RT-PCR DNA assay, were used in monoplex and multiplex runs in order to detect and identify the carbapenemases from microbial cultures. The LightMix® modular carbapenemase kits were run on a Cobas z 480 Analyser (open channel), (Roche Diagnostics International Ltd. 6343 Rotkreuz, Switzerland).

LightMix® Universal Color Compensation Hexaplex was used before the first run after Roche

manual indication. (TIB MOLBIOL Syntheselabor GmbH | Eresburgstr. 22-23 | D-12103 Berlin | Germany) (12,13). Depending on our targets, we used the optimal choice of dye and detection channel combinations.

The following targets were chosen: $bla_{KPC}$, $bla_{NDM}$, $bla_{GES}$, $bla_{IMP}$ and $bla_{OXA-48}$ CR genes and mcr-1 for colistin resistance. We used the LightMix® Modular kit for the detection of the following carbapenemases: OXA-48 members (162, 163, 244, 245, 247, 204 ,181, 232), KPC (types 1-11), GES (1-9 and 11), IMP (detects most described IMP variants except IMP-9,16,18, and 25 types) and NDM (NDM-1 to NDM-13, NDM-16, NDM-17) carbapenemases. The amplification product was detected with a specific labeled hydrolysis probe (14). The LightCycler® Multiplex DNA Master is an optimized mix used with hydrolysis probes in the reaction mix. Version 06 (2017) was used (12).

Two reaction mixes were optimized for multiplex real-time PCR analysis of DNA. First mix contained primers for GES, NDM, and KPC genes detection. The second reaction mix contained two primers, one for OXA-48 and the other for IMP gene detection. The detection mcr-1 mix was performed separately on the LightCycler® 480 Multiwell Plate 96.

The reaction mixture consisted of: 10.5 $\mu$l PCR-grade water (Roche Master kit) +0.5 $\mu$l Reagent mix (parameter specific reagents containing primers and probes) +4.0 $\mu$l Roche Master + 5 µl of sample or control to each well. The final volume of the reaction was 20 $\mu$l.

PhHV was used as a spiked extraction control to monitor the integrity of the DNA extracted from KP cultures and the presence of reaction inhibitors.

A protocol consisting of three program steps was applied: denaturation at 95°C/5 min/1 cycle, cycling for 45 cycles of 95°C/5 s, 60°C/15 s and 72°C/15 s and cooling at 40°C / 30 s/1 cycle. The execution time of the run was approximately 70 minutes.

We used one specific positive control (amplified at crossing point (Cp)<37) available with the kit for each gene tested and three negative test controls (NTC showed no signal).

The Cobas® 4800 software 2.2.0 variant was used to interpret the results. Results were analyzed using color compensation and we used specific reading channels to view the results for resistance genes: the channel 640 for GES, 530 for NDM, 610 for KPC, 580 for OXA-48 and 640 for IMP. The reading channel for MCR-1 gene was 640. The limit detection is 10 genetically equivalent copies. The reaction sensitivity and specificity, declared by the manufacturer, were 100% with 100% NPV (negative predictive value) and 93.3% PPV (positive predictive value).

***Statistical Analysis***

Quantitative continuous variables (age, hospitalization length) were described using arithmetic mean for Gaussian distribution of data or median with interquartile interval (Q1= lower quartile; Q3=upper quartile) for distributions with deviations from Gaussian distribution. Qualitative variables were summarized by case numbers and percentages (%).

Real-time PCR was considered as a gold standard method. The results of CDT test and RT-PCR were compared using the nonparametric McNemar's test. Sensitivity, specificity, and accuracy of prediction with associated 95% confidence intervals (CI) were estimated.

All statistical tests used in the data analysis were two-sided with a significance level chosen at $\alpha = 0.05$. Statistical analysis was performed in R software version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria).

## Results

A number of 47 *Klebsiella pneumoniae* (KP) were isolated. Considering the distribution of the

MIC value, 61.70% (29/47) of the KP were carbapenem-susceptible and 38.30% (18/47) were carbapenem-resistant. Table 1 shows the MIC values for three carbapenems in the carbapenem-resistant KP-group.

The carbapenem-sensitive group (61.70%; 29/47) comprise KP (31.91%; 15/47) with other resistance mechanisms against β-lactams (e.g., extended spectrum beta-lactamase (ESBLs)) and wild (natural) KP phenotypes (29.79%; 14/47). All these isolates had the meropenem MIC≤0.25 μg ml-1 and were negative at the mCIM phenotypic test designed to highlight the carbapenemase-producers (data not shown).

We found no significant difference in age and gender distribution (p≥0.05), patients whose blood cultures were positive for KP with CR, ESBL or wild phenotype, having a similar length of hospitalization (Table 2).

The CDT showed the following results: 55.56% (10/18) isolates showed no synergy between meropenem and meropenem + inhibitors and temocillin not-susceptible, suggesting OXA-48 production. One isolate (5.55%), showed a noninterpretable profile; 38.89% (7/18) strains produced the KPC enzyme, showing synergism between meropenem and meropenem - boronic acid.

The following results were obtained using the LightCycler® Multiplex DNA Master: 50% (9/18) were KPC and OXA-48 co-producing strains; 44.45% (8/18) isolates were KP harboring $bla_{OXA-48}$ and only one isolate (5.55%; 1/18) was a KPC producer. Positive results were amplified at Cp<37. The samples with Cp >37 were

**Table 1. Measured MIC values in the carbapenem-resistant KP-group**

| KP Number | MRP | SIR | IMI | SIR | ERT | SIR |
|---|---|---|---|---|---|---|
| KP01 | 2 | I | 2 | I | ≥8 | R |
| KP02 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP03 | ≥16 | R | 4 | I | ≥8 | R |
| KP04 | ≥16 | R | 8 | I | ≥8 | R |
| KP05 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP06 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP07 | ≥16 | R | 4 | I | ≥8 | R |
| KP08 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP09 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP10 | 2 | I | 2 | I | 4 | R |
| KP11 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP12 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP13 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP14 | 4 | I | 8 | I | ≥8 | R |
| KP15 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP16 | 2 | I | ≥16 | R | ≥8 | R |
| KP17 | ≥16 | R | ≥16 | R | ≥8 | R |
| KP18 | ≥16 | R | ≥16 | R | ≥8 | R |

MIC= minimum inhibitory concentration (μg/ml); SIR= sensible; intermediar; resistant; MRP = meropenem; IMI = imipenem; ERT = ertapenem.

**Table 2. Demographic and clinical characteristics of the hospitalized patients with positive blood culture for *Klebsiella pneumoniae* (KP)**

| Variables | CR KP-group ($n_1$ = 18) | ESBL KP-group ($n_2$ = 15) | WILDS KP-group ($n_3$ = 14) | p-value |
|---|---|---|---|---|
| Age, years | 60.61 ± 12.19 | 70.60 ± 10.51 | 61.71 ± 13.30 | 0.05 |
| Gender | | | | 0.836 |
| Male | 9 (50.00) | 9 (40.00) | 8 (42.86) | |
| Female | 9 (50.00) | 6 (60.00) | 6 (57.14) | |
| Hospitalisation length (days) | 26 [20.25, 34.50] | 20 [13.50, 27.00] | 26 [16.50, 29.25] | 0.2129 |
| Deaths | 10 (55.56 | 5 (33.33) | 4 (28.57) | 0.2416 |

Data are expressed as arithmetic mean ± standard deviation, median [IQR], IQR = interquartile interval or absolute frequencies (percentages calculated from the size of group); CR = carbapenem-resistant KP-group, ESBL = extended spectrum beta-lactamase; WILD = natural phenotype

considered negative. All isolates were negative for the MCR-1 gene.

The results of the multiplex run also recorded amplification curves with Cp> 37 for six isolates that required confirmation of the presence of *bla*$_{GES}$ and *bla*$_{IMP}$ genes by performing monoplex runs (table 3). The monoplex run tests for both LightMix® Modular GES and IMP kits, were negative for all 18 KP tested.

The phenotypic results of eight isolates (KP 3, KP 5, KP 8-11, KP 16, KP 18) were confirmed by molecular LightCycler® Multiplex DNA assay. The phenotypic result was the opposite for one isolate (KP 7). The phenotypic test of eight isolates (KP 2, KP 4, KP 6, KP 12-15 and KP 17) which were simultaneously KPC and OXA-48 producers, indicated the presence of only one CR gene (Table 4).

In the CR-KP group, RT-PCR identified the simultaneous presence of KPC and OXA-48 genes in 9 cases (Figure 1).

The 29 carbapenem-sensitive isolates analyzed as a negative group-control were negative with both phenotypic tests and these results were confirmed with LightCycler® Multiplex DNA assay. The results of Table 5 highlighted concordant results between CDT and RT-PCR concerning KPC (McNemar test, p=0.6171), but discordant results regarding OXA-48 (p=0.0412). The results also showed a higher specificity of combined disk test compared with multiplex RT-PCR for KPC in CR-KP group (Sp= 88%, 95% CI: 47% − 100%) and high positive predictive and negative values (PPV = 86%, 95% CI: 42% – 100%, NPV = 70%, 95% CI: 35% −93%). Concerning OXA-48 we obtained the following results regarding PPV = 100%, 95% CI: 69% − 100% and NPV = 14%, 95% CI: 0.00% − 58%.

## Discussions

In 2011 21 selected European nations, Romania included, were monitored in an antimicrobial surveillance program. In the published report of the program named Regional Resistance Surveillance Study, the authors showed the presence of CRE (carbapenem-resistant *Enterobacteriaceae*) in 10 countries (including Romania) at

**Table 3. Results of LightCycler multiplex first run.**

| KP NUMBER | bla$_{OXA-48}$ | bla$_{KPC}$ | bla$_{IMP}$ | bla$_{GES}$ | bla$_{NDM}$ | mcr-1 |
|---|---|---|---|---|---|---|
| KP01 | 1 | 1 | 0 | 0 | 0 | 0 |
| KP02 | 1 | 1 | 0 | 2 | 0 | 0 |
| KP03 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP04 | 1 | 1 | 2 | 0 | 0 | 0 |
| KP05 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP06 | 1 | 1 | 0 | 0 | 0 | 0 |
| KP07 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP08 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP09 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP10 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP11 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP12 | 1 | 1 | 0 | 2 | 0 | 0 |
| KP13 | 1 | 1 | 0 | 2 | 0 | 0 |
| KP14 | 1 | 1 | 2 | 0 | 0 | 0 |
| KP15 | 1 | 1 | 2 | 0 | 0 | 0 |
| KP16 | 1 | 0 | 0 | 0 | 0 | 0 |
| KP17 | 1 | 1 | 0 | 0 | 0 | 0 |
| KP18 | 0 | 1 | 0 | 0 | 0 | 0 |

0= gene not detected; 1=gene detected, 2= gene detected with confirmation required

**Tabel 4. Results of CDT and RT-PCR tests:**

| KP NUMBER | CDT PROFILE | TEM | RT-PCR |
|---|---|---|---|
| KP01 | NI | 17 | KPC+OXA-48 |
| KP02 | KPC | 17 | KPC+OXA-48 |
| KP03 | OXA | 9 | OXA-48 |
| KP04 | OXA | 9 | KPC+OXA-48 |
| KP05 | OXA | 9 | OXA-48 |
| KP06 | KPC | 9 | KPC+OXA-48 |
| KP07 | KPC | 9 | OXA-48 |
| KP08 | OXA | 9 | OXA-48 |
| KP09 | OXA | 9 | OXA-48 |
| KP10 | OXA | 9 | OXA-48 |
| KP11 | OXA | 9 | OXA-48 |
| KP12 | KPC | 24 | KPC+OXA-48 |
| KP13 | KPC | 19 | KPC+OXA-48 |
| KP14 | OXA | 10 | KPC+OXA-48 |
| KP15 | KPC | 16 | KPC+OXA-48 |
| KP16 | OXA | 9 | OXA-48 |
| KP17 | OXA | 9 | KPC+OXA-48 |
| KP18 | KPC | 16 | KPC |

TEM: temocillin-diameter of inhibition zone (mm); NI: noninterpretable test

0.9–38.8% rates, the rate being highest in Poland and Italy (32.5–38.8%) (15).

Romania is a member of the European Antimicrobial Resistance Surveillance Network (EARSNet). Organized at national level, there is a sentinel surveillance system of nosocomial infections which also includes CR-KP. The nosocomial reports do not include the carbapenem resistance mechanisms or type of carbapenemases produced. Our clinical hospital is part of this system. Starting with 2018, we also report the MICs of *Klebsiella pneumoniae*, *Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus* and *Enterococcus faecalis* isolated in blood-cultures, in the WHONET software, developed by OMS for the AMR surveillance program.

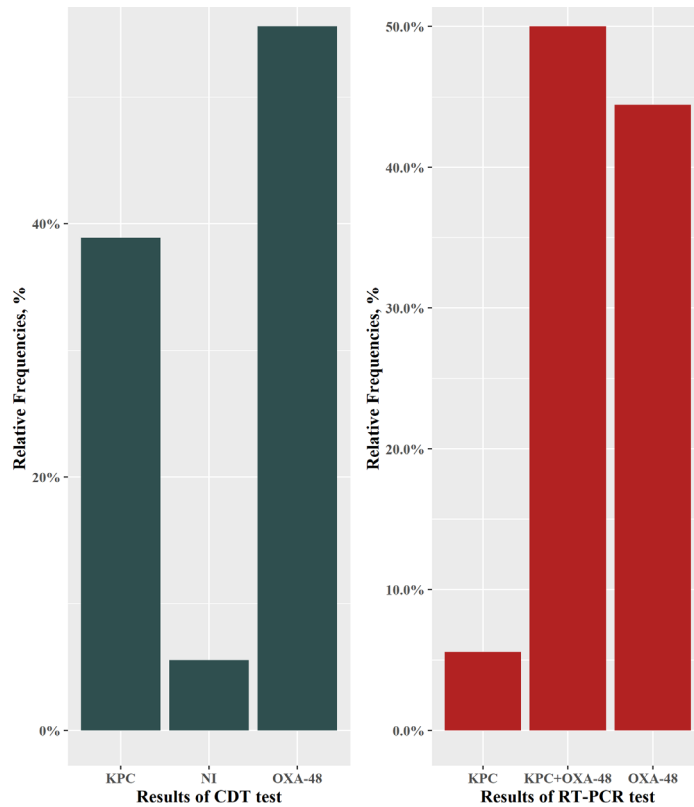


**Fig. 1. Distribution of bla$_{KPC}$, bla$_{NDM}$, bla$_{GES}$, bla$_{IMP}$ and bla$_{OXA-48}$ CR genes in CR KP-group by CDT and RT-PCR tests; NI= noninterpretable profile.**

**Table 5. Performance indicators assessment of phenotypic (CDT) determination compared to molecular (multiplex RT-PCR) in all *Klebsiella pneumoniae* strains and CR-KB group**

| Carbapenem resistance genes | RT-PCR | | Apparent relative frequency (%) [95% CI] | Se [95% CI] | Sp [95% CI] | Accuracy [95% CI] | McNemar test |
|---|---|---|---|---|---|---|---|
| | positive | negative | | | | | |
| **All sample (n=46)** | | | | | | | |
| **OXA-48** | 10 | 0 | 22 [11, 36] | 62 [35, 85] | 100 [88, 100] | 87 [74, 95] | p-value = 0.0412 |
| **KPC** | 6 | 1 | 15 [6, 29] | 67 [30, 93] | 97 [86,100] | 91 [79, 98] | p-value = 0.6171 |
| **IMP** | 0 | 0 | 0 [0, 8] | NA | 100 [92,100] | 100 [92,100] | NA |
| **GES** | 0 | 0 | 0 [0, 8] | NA | 100 [92,100] | 100 [92,100] | NA |
| **NDM** | 0 | 0 | 0 [0, 8] | NA | 100 [92,100] | 100 [92,100] | NA |
| **MCR-1** | 0 | 0 | 0 [0, 8] | NA | 100 [92,100] | 100 [92,100] | NA |
| **CR-KP group ($n_1$=18)** | | | | | | | |
| **OXA-48** | 10 | 0 | 59 [33, 82] | 62 [35, 85] | 100 [2, 100] | 65 [38 86] | p-value = 0.0412 |
| **KPC** | 6 | 1 | 41 [18,67] | 67 [30, 93] | 88 [47,100] | 76 [50, 93] | p-value = 0.6171 |
| **IMP** | 0 | 0 | 6 [0, 29] | NA | 100 [80,100] | 100 [80,100] | NA |
| **GES** | 0 | 0 | 0 [0, 20] | NA | 100 [80,100] | 100 [80,100] | NA |
| **NDM** | 0 | 0 | 0 [0, 20] | NA | 100 [80,100] | 100 [80,100] | NA |
| **MCR-1** | 0 | 0 | 0 [0, 20] | NA | 100 [80,100] | 100 [80,100] | NA |

n=46 samples; Accuracy = Correctly classified proportion; Se=Sensitivity; Sp=Specificity; NA=not available due to lack of cases with studied genes; RT-PCR=golden standard; the KP strain with noninterpretable test at CDT test was excluded from concordance analysis.

The first characterization of CRE isolated in Romania was published in 2013. The researchers analyzed nine CR-isolates collected in an emergency university hospital located in the center of Romania. It described the local distribution of carbapenemase encoding genes: $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181.}}$ The CRE analyzed bacteria comprised five CR-KP, three of them were OXA-48 producing strains, one was harboring NDM gene and another was NDM and OXA-181 co-producing KP(16). NDM carbapenemase was not detected among our tested strains. The second study analyzed the distribution of CRE in two Romanian intensive care units of two hospitals from Bucharest, a survey during one year (2011–2012) and reported the genes $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ in *Enterobacterales* (17). In 2015 a published study evaluated the emergence of carbapenem-resistant *Enterobacterales* among the strains isolated from hospitalized patients in the National Institute of Infectious Diseases, Bucharest (NIID). The authors reported OXA-48-like as the most frequent carbapenemase isolated during the six months survey (18). Lixandru and all presented the first description of KP strains harboring $bla_{\text{KPC-2}}$ and $bla_{\text{VIM-1}}$ genes in Romania (19). Another study described the emergence of CRE strains in a Romanian hospital located in the North of the country, where KPC was the main carbapenemase detected (20).

The present study provides a twelve-month retrospective analysis about the occurrence of KP-CR isolated from blood-cultures from hospitalized patients in the Romanian regional clinical hospital. We studied 303 (14.91%) positive blood cultures (without duplicates) out of 2032 pairs of blood cultures collected consecutively from hospitalized patients. We identified 153 GNB out of which 111 were *Enterobacterales* members. A number of 18 strains were identified as CR-KP(7). To our knowledge, we performed, for the first time in Romania, the LightCycler®

Multiplex DNA assay in order to detect CR-KP and to identify the type of carbapenemases. Also, we first reported CR-KP isolates carrying both KPC and OXA-48-like carbapenemase genes in the central and north-west Romanian regions.

Lopes et al. analyzed the carbapenemase-producing KP in a hospital from northern Portugal and compared their results with the CR-KP circulating in the rest of the country. KPC-2 was the predominant carbapenemase type in their region, followed by OXA-48. The predominant carbapenemase type was KPC-3 in the rest of the territory (21). Our study revealed the presence of the same classes of carbapenemases but with OXA-48 being found most frequently.

A group of researchers observed in their study that the presence of carbapenem resistance may not be detected if the MIC for meropenem is between 0.25-0.5 mg / L. This is especially related to the presence of the OXA gene (22). Our study demonstrates the presence of OXA-48 producing KP in the hospital, which represent almost half of the analyzed CR-KP (44.45%). A reliable detection became mandatory, required for preventing further spread.

This study showed that, all ertapenem resistant isolates (17/18 isolates MIC≥8 µg/ml, one MIC 4 µg/ml) were carbapenemase-producing KP. EUCAST guidelines version 2.0 (July 2017), consider ertapenem as having high sensitivity but low specificity because an isolate with ESBLs and AmpC can be ertapenem-resistant (9). Therefore, we consider it important to test ertapenem-resistant Klebsiella pneumoniae isolates by CDT (or other phenotypic methods) in order to detect carbapenemase-producing strains. CDT contains specific inhibitors of carbapenemase activity and differentiates between carbapenemase classes and non-carbapenemases (ESBL and/or AmpC plus porin loss), if KP produces a single type of carbapenemase (9). One of the possible reasons why the CDT test missed the carbapenemases might be the fact that many strains co-harbored two carbapenemase genes. In our researched KP isolates, eight strains were simultaneously KPC and OXA-48 producers but CDT identified only one carbapenemase. If CDT results were inconclusive (e.g., the OXA-48-like producers, the simultaneous presence of two or more carbapenem resistance mechanisms, strains co-harbored two carbapenemase genes), the method of choice in this instance was molecular testing (9,23).

Oviaño et al. evaluated in 2016 the LightMix® modular carbapenemase kits declaring an overall sensitivity and specificity of 99% and 100%, respectively. The conclusion of their research was that these kits are useful for testing colonized and septic patients (24). Our results were in line with findings obtained by Oviaño et al. concerning high values of specificity, positive and negative predictive values obtained for $bla_{KPC}$ gene.

Recently, a Japanese research team developed a multiplex RT-PCR assay with the Cobas® z480 analyzer. Yoshioka et al. used two combinations of carbapenemases detection primers, as we did, but selected partially different primers. They concluded that the assay appropriately differentiated the reference or clinical strains harboring each carbapenemase gene without cross reactivity (25).

LightMix® Modular carbapenemases Kits detect the main classes of carbapenemases with a high level of accuracy. Running a multiplex test would save time and money.

### *Limits*

Our analyses were performed in only one clinical hospital comprising a limited number of target genes. Detection of other CR mechanisms like cephalosporinase over-expression, porin mutations, and efflux mechanism was not investigated.

## Conclusions

Multiplex real-time PCR assay detected and differentiated with precision in one run time the

carbapenemase producing microorganisms with complex genetic mechanisms harboring more than one different carbapenemase gene. This method is robust and useful for rapid diagnostic and patient screening, allowing the choice of specific epidemiological measures to avoid the MDR spread. To differentiate between five genes, it was required to perform two sets per run, which was disadvantageous.

The results of this study are important for epidemiologic evaluation of carbapenem-resistant *Klebsiella pneumoniae* strains. It demonstrates the presence of the $bla_{\text{OXA-48-like}}$ and the $bla_{\text{KPC}}$ genes and can be representative considering the circulation of these carbapenemase harboring strains in the center and north-west regions of Romania. Our future aim is to determine all the genes involved in carbapenem resistance and to compare the strains phenotypes and molecular resistance profile between different Romanian areas.

## Abbreviations

carbapenem-resistant *Klebsiella pneumoniae* (CR-KP)
*Klebsiella pneumoniae* (KP)
Gram-negative bacilli (GNB).
difficult-to-treat phenotype (DTR)
antimicrobial susceptibility tests (AST)
the combination disks test (CDT)
minimum inhibitory concentration (MIC)
real-time polymerase chain reaction (RT-PCR),
carbapenem resistance (CR)
Surveillance of antimicrobial resistance (AMR)
isolates identification (ID)
the Clinical and Laboratory Standards Institute (CLSI)
European Committee on Antimicrobial Susceptibility Testing (EUCAST)
the negative control (NTC)
crossing point (Cp)
negative predictive value (NPV)
positive predictive value (PPV).
confidence intervals (CI)
sensible; intermediar; resistant (SIR)
meropenem (MRP)
imipenem (IMI)
ertapenem (ERT)
carbapenem-resistant *Enterobacteriaceae* (CRE)

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## Author contributions

MT, MI, SLP, RT and MDG contributed to the conceptualization, methodology, data curation, investigation, visualization, drafting, editing and reviewing of the manuscript. MC and SMP contributed to the investigation and data curation of the manuscript. MT and MI contributed to the literature search and critical revision of the manuscript. LMJ contributed as the supervisor of work, of literature, references, the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. MT and MI confirm the authenticity of all the raw data.

## Conflicts of Interest

There were none competing interest.

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