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Performance Assessment of Three Phenotypic Tests for Carbapenemase Detection in *Enterobacterales*

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Carbapenemase-producing *Enterobacterales* (CPE) pose a significant threat in hospital settings—especially in intensive care units—from both therapeutic and epidemiological perspectives. Rapid identification is crucial. The aim of this study was to evaluate three phenotypic methods used in routine diagnostics. The study included 56 clinical isolates of carbapenem-resistant *Enterobacterales* collected from patients hospitalized at the University Clinical Center (UCC) Niš. Among these isolates, 52 were confirmed as carbapenemase producers, while four lacked carbapenemase genes. Genotypic detection was performed using Multiplex PCR targeting the *bla*KPC, *bla*VIM, *bla*NDM, and *bla*OXA-48 genes. The evaluated phenotypic methods included the NG-Test Carba 5, the RAPIDEC Carba NP test (RCNP), and a commercial combination disk test (CDT) - KPC, MBL, and OXA-48 Confirm Kit: Carbapenemases. Multiplex PCR revealed: 2 KPC producers; 24 NDM producers; 16 OXA-48-like producers; 10 isolates producing both NDM and OXA-48 enzymes. One isolate of *Enterobacter cloacae* was identified as a co-producer of NDM, KPC, and OXA-48 enzymes, and one isolate of

Klebsiella pneumoniae as a co-producer of NDM and KPC enzymes. The sensitivity and specificity of the NG-Test Carba 5 were 98.08%/100.00%. In the Carba NP test, after 120 minutes, sensitivity and specificity were 90.38% and 100%, respectively. For the CDT method, the sensitivity and specificity for detecting metallo-β-lactamases (MBL) using dipicolinic acid (DPA) were 80.56% and 100%, respectively, while for detecting class D carbapenemases using temocillin they were 95.65% and 100%, respectively. The best results in detecting specific classes of carbapenemases were achieved with the NG-Test Carba 5 and the CDT method. These methods could be employed for rapid and reliable detection of carbapenemases in routine diagnostics.

Keywords: Carbapenemase production; *Enterobacterales*; phenotypic methods for carbapenemase detection

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Procena performansi tri fenotipska testa za detekciju karbapenemaza kod enterobakterija

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Enterobakterije koje proizvode karbapenemaze (CPE) predstavljaju značajan terapijski i epidemiološki problem u bolničkom okruženju, posebno na odeljenjima intenzivne nege. Brza identifikacija ovih mikroorganizama je od ključnog značaja. Cilj ove studije bio je procena tri fenotipske metode koje se koriste u rutinskoj dijagnostici. Studija je obuhvatila 56 kliničkih izolata enterobakterija rezistentnih na karbapeneme, izolovanih kod pacijenata hospitalizovanih u Univerzitetskom kliničkom centru (UKC) Niš. Kod 52 izolata potvrđena je produkcija karbapenemaza, dok četiri izolata nisu imala gene za karbapenemaze. Genotipska detekcija sprovedena je Multiplex PCR metodom, targetirajući *blaKPC*, *blaVIM*, *blaNDM* i *blaOXA*-48 gene. Fenotipske metode koje su analizirane uključivale su NG-Test Carba 5, RAPIDEC Carba NP test (RCNP) i komercijalni kombinovani disk test (CDT) - KPC, MBL i OXA-48 Confirm Kit: Carbapenemases. Multiplex PCR je pokazao sledeću distribuciju karbapenemaza: 2 izolata su bili KPC produktori, 24 su produkovala NDM, 16 su bili produktori OXA-48 enzima, dok je kod 10 izolata detektovana istovremena produkcija NDM i OXA-48 enzima. Takođe, jedan izolat *Enterobacter cloacae* identifikovan je kao koproduktor

NDM, KPC i OXA-48 enzima, dok je jedan izolat *Klebsiella pneumoniae* istovremeno produkovao NDM i KPC enzime. Osetljivost (senzitivnost, Se) i specifičnost (Sp) NG-Test Carba 5 iznosile su 98,08% i 100%. Kod Carba NP testa, nakon 120 minuta, Se i Sp su bile 90,38% i 100%. Kod CDT metode, Se i Sp za detekciju metalo-β-laktamaza (MBL) pomoću dipikolinične kiseline (DPA) bile su 80,56% i 100%, dok su za detekciju klase D karbapenemaza pomoću temocilina iznosile 95,65% i 100%. Najbolje rezultate u detekciji specifičnih klasa karbapenemaza pokazali su NG-Test Carba 5 i CDT metoda. Ove metode bi mogle biti korišćene za brzu i pouzdanu detekciju karbapenemaza u rutinskoj dijagnostici.

Ključne reči: Produkcija karbapenemaza; enterobakterije; fenotipske metode za detekciju karbapenemaza

INTRODUCTION

Carbapenemase-producing *Enterobacterales* (CPE) cause infections with limited treatment options and are associated with high morbidity and mortality, particularly in patients in intensive care units. The emergence of antibiotic resistance is often due to natural factors; however, the primary contributor to this issue is the misuse of antimicrobial agents. The presence of such strains in hospital settings, especially in intensive care units, poses a significant threat not only from a clinical perspective but also from an epidemiological standpoint, where the spread of carbapenemases and potential outbreaks could lead to major health problems for hospitalized patients (1).

Carbapenem resistance in *Enterobacterales* involves two types of mechanisms. One, which is less significant, is based on the production of ESBL and AmpC enzymes combined with efflux mechanisms and alterations in porin channels. The other, much more significant, is the production of carbapenemases. This mechanism results not only in high levels of resistance but also enables the rapid spread and colonization of these enzyme-producing strains in hospital settings. According to Ambler's classification, carbapenemases are divided into three major classes: Ambler class A (KPC), class B (metallo-β-lactamases – VIM, NDM, and IMP), and class D (OXA-48-like) (2). Considering the significance of carbapenemase production, the rapid detection of CPE is crucial for infection control, preventing hospital outbreaks, and optimizing antibiotic therapy for the infections they cause. Although PCR is regarded as the reference method for carbapenemase detection, phenotypic tests are much more suitable for routine use (3).

There are several phenotypic tests for detecting carbapenemase production: biochemical (colorimetric) tests (e.g. Carba NP test), modified Hodge test [MHT], carbapenem inactivation method (mCIM) (4), rapid multiplex immunoassay (e.g NG-Test Carba 5) (5), MIC MBL test (6), combined disc test (CDT) (7), synergistic tests- boronic acid and ethylenediaminetetraacetic acid (EDTA) synergy tests (8). For detecting carbapenemase on *Enterobacterales*, especially for infection control purposes and public health purposes, EUCAST proposed the use of combination disk testing, Carba NP test, mCIM assays, Carbapenem Inactivation Method, detection of carbapenem hydrolysis with MALDI-TOF and lateral flow assays (9). Our study compared the performance of three selected methods for detecting carbapenemase production in *Enterobacterales* with reduced susceptibility to carbapenems.

Materials and Methods

Bacterial Isolates

Identification and Selection

The study was conducted at the Microbiology Center of the Institute of Public Health in Niš. The study included 56 primary isolates of carbapenem-resistant *Enterobacterales* (CRE) obtained from samples of patients hospitalized at UCC Niš. Bacterial isolates were isolated and identified using standard bacteriological methods. Species-level identification and carbapenem susceptibility testing were performed using the automated Vitek 2 Compact system (BioMerieux, Marcy l'Etoile, France). Among the tested isolates*, Klebsiella pneumoniae (K. pneumoniae*) was the most prevalent (35 isolates), followed by *Enterobacter cloacae (E. cloacae)* (11 isolates), *Serratia marcescens (S. marcescens),* (3 isolates), *Enterobacter aerogenes* (*E. aerogenes*) (2 isolates), *Citrobacter freundii (C. freundii)* (2 isolates), and one isolate each of *Escherichia coli (E. coli), Morganella morganii (M. morganii*) and *Proteus mirabilis (P. mirabilis).*

Multiplex PCR was used to detect the *bla*KPC, *bla*VIM, *bla*NDM, and *bla*OXA-48 genes. This analysis revealed: 24 NDM producers (*10 K. pneumoniae, 8 E. cloacae, 2 C. freundii, 1 E. coli, 1 S. marcescens, 1 M. morganii,* and 1 *E. aerogenes*), 2 KPC producers (both *K. pneumoniae*), 16 OXA-48-like producers (all *K. pneumoniae*), and 10 isolates producing both NDM and OXA-48 enzymes (7 *K. pneumoniae*, 2 *E. cloacae*, and 1 *E. aerogenes*). Additionally, one isolate of *E. cloacae* was identified as a co-producer of NDM, KPC, and OXA-48 enzymes, and one isolate of *K. pneumoniae* was identified as a co-producer of NDM and KPC enzymes. Isolates in which carbapenemase production was not confirmed: *S. marcescens* (2), *P. mirabilis* (1), and *E. aerogenes* (1). *K. pneumoniae* BAA 1705 (KPC-2), *K. pneumoniae* NCTC 13443 (NDM-1), *K. pneumoniae* NCTC 13440 (VIM) and *K. pneumoniae* NCTC 13442 (OXA-48) were used as positive controls and *E. coli* ATCC 25922 were used as a negative control.

Phenotypic Methods for Detecting Carbapenemase Production

Combined Disk Test (KPC, MBL, and OXA-48 Confirm Kit: Carbapenemases-RCDT)

The KPC, MBL, and OXA-48 Confirm Kit: Carbapenemases (Rosco Diagnostica, Denmark) was used as the combined disk test. A 10 μg meropenem disk was placed 30 mm apart from disks containing meropenem/dipicolinic acid (MBL inhibitor), meropenem/boronic acid (KPC inhibitor), meropenem/cloxacillin (AmpC inhibitor), and temocillin. After 24-hour incubation at 35°C, the inhibition zones for each tested disk were measured. The CDT test interpretation was performed according to the manufacturer's instructions. For temocillin, isolates were considered positive if

resistance to temocillin (≤11 mm) was detected, provided there was no difference greater than 3 mm in the inhibition zones between meropenem alone and its combination with DPA, cloxacillin, and boronic acid. In the CDT test with EDTA**,** a ≥7 mm difference in the inhibition zone between imipenem and imipenem-EDTA (10 µg/750 µg) was considered a positive result.

Representation of the combined disk test for detecting carbapenemase production are showed in figure 1.

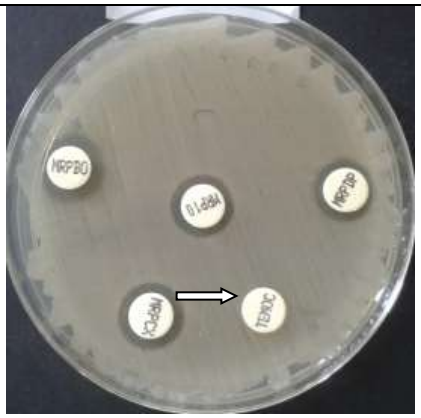
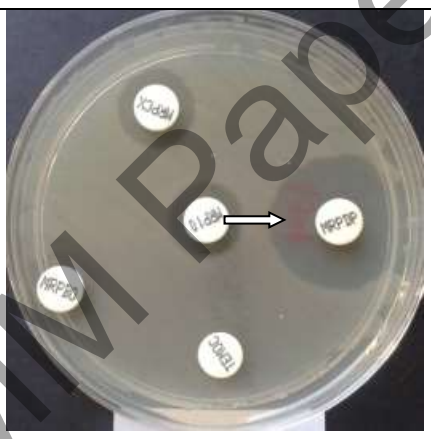
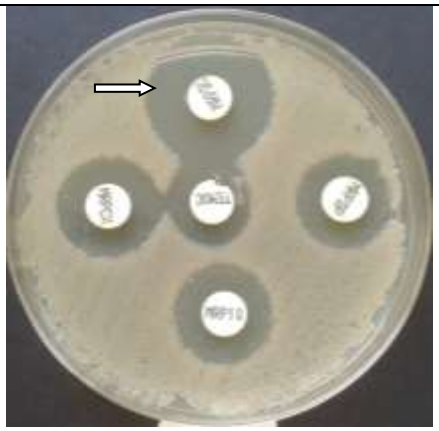
	
OXA-48 positive isolate – No inhibition zone around the temocillin disc ([5]TEMOC), and no difference in the inhibition zone between [1]MRP 10 and the combinations [2]MRP BO, [3]MRP DP, and [4]MRP CX.	Carbapenemase-negative isolate **–** No differences in the inhibition zone between MRP 10 and the combinations [2]MRP BO, [3]MRP DP, and [4]MRP CX.
	
NDM-positive isolate – Difference in the inhibition zone between [1]MRP and [3]MRP DP >5 mm.	KPC-positive isolate – Difference in the inhibition zone between [1]MRP and [2]MRP BO >5 mm

Figure 1. Representation of the combined disk test for detecting carbapenemase production.

[1]MRP 10 – meropenem, [2]MRP BO – meropenem-boronic acid, [3]MRP DP – meropenem-dipicolinic acid, [4]MRP CX – meropenem-cloxacillin, [5]TEMOC- temocillin

Colorimetric Test- RAPIDEC Carba NP (RCNP)

The colorimetric test for carbapenemase detection is a classic acidimetric assay with a colorimetric endpoint, where the phenol red indicator turns yellow upon carbapenem hydrolysis. For this purpose, the commercial RAPIDEC® CARBA NP test (bioMérieux, France) was employed. This test is designed for the rapid detection of carbapenemases in Gram-negative bacteria such as *Enterobacterales*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. It is based on detecting the hydrolysis of the β-lactam ring in the imipenem molecule. The hydrolysis leads to acidification of the medium, which causes a visible color change in the pH indicator (phenol red). Results are interpreted after 30 minutes and again after 2 hours; the absence of a color change after 2 hours is considered a negative result. Figure 2. illustrates the colorimetric test for carbapenemase detection.

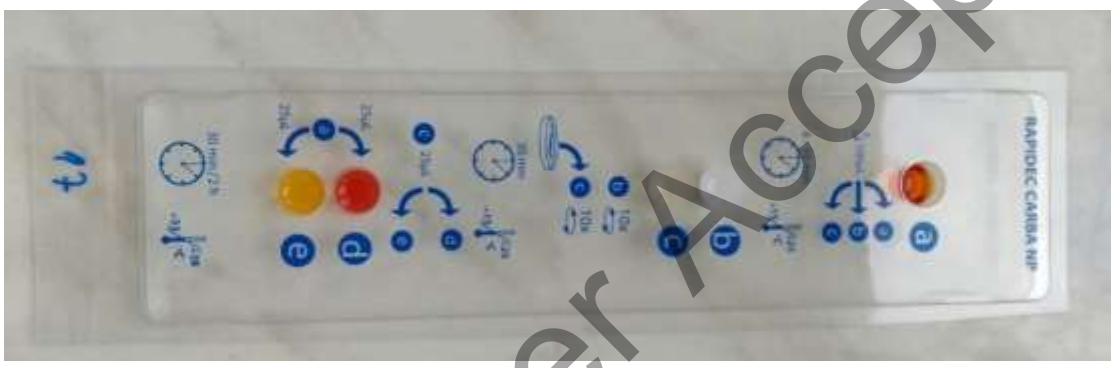


Figure 2. Positive result of RAPIDEC Carba NP test

In Vitro Multiplex Immunoassay – NG-Test CARBA 5 (NG-Biotech)

NG-Test CARBA 5 Protocol Description

A 1-µL loop was used to collect three bacterial colonies, which were then suspended in a 1.5 mL microcentrifuge tube prefilled with five drops of extraction buffer, as provided by the manufacturer. Following a brief vortexing step, 100 µL of the resulting suspension was transferred into the sample well (S) of the test cassette using the manufacturer-supplied disposable transfer pipette. After 15 minutes, the cassette was visually examined for the appearance of control and test lines. The figure 3. shows a positive test for different types of carbapenemases.

Figure 3. Positive NG-Test CARBA 5 test for different types of carbapenemases

*C-control line ;K-KPC; O-OXA-48; V-VIM; I-IMP; N-NDM

Statistical Data Analysis

The validity of phenotypic tests was assessed and expressed in terms of sensitivity and specificity, using PCR-based detection of resistance genes as the gold standard. Sensitivity (the proportion of carbapenemase-producing isolates correctly identified) and specificity (the proportion of carbapenemase-negative isolates correctly distinguished) were determined for each method used,as well as positive predictive value (PPV) and negative predictive value (NPV) (10). The data is presented according to the principles of descriptive statistics, and hypothesis testing was conducted using appropriate tests in the MedCalc statistical software – https:// www.medcalc. org/ calc/ diagnostic test.php.

RESULTS

The susceptibility of carbapenemase-producing *Enterobacterales* isolates to antimicrobial agents was determined using the Vitek2 method. The carbapenem susceptibility of carbapenemase-producing isolates, as well as positive and negative controls, is presented in Table1. The isolates were grouped according to bacterial species and resistance genes.The susceptibility of carbapenemase-negative isolates is shown in Table 2.

Table 1. Carbapenem Susceptibility of Carbapenemase-Producing Isolates, Positive and Negative Controls

Species name	Gen of resistance	Number of isolates	Ertapenem MIC (µg/ml)	Imipenem MIC (µg/ml)	Meropenem MIC(µg/ml)
K. pneumoniae	bla_{NDM}	10	4- ≥8	0.25- ≥16	1- ≥16
	bla_{NDM} / bla_{OXA-48}	7	4- ≥8	0.25- ≥16	1- ≥16
	bla_{OXA-48}	16	4- ≥8	0.25- ≥16	1- ≥16
	bla_{KPC}/bla_{NDM}	1	≥8	≥16	≥16
E. aerogenes	bla_{NDM}	1	4- ≥8	0.25- ≥16	1- ≥16
	bla_{NDM} / bla_{OXA-48}	1	4- ≥8	0.25- ≥16	1- ≥16
E. cloacae	bla_{NDM}	8	4- ≥8	0.25- ≥16	1- ≥16
	bla_{NDM} / bla_{OXA-48}	2	≥8	≥16	≥16
	bla_{KPC}/ bla_{NDM} / bla_{OXA-48}	1	≥8	≥16	≥16
S. marcescens	bla_{NDM}	1	≥8	0.5	1.0
M. morganii	bla_{NDM}	1	1.0	1.0	4.0
E. coli	bla_{NDM}	1	≥8	≥16	≥16
Pozitivne kontrole					
K. pneumoniae NCTC 13440	bla_{VIM}	56	≥8	≥16	≥16
K. pneumoniae NCTC 13443	bla_{NDM}		≥8	≥16	≥16
K. pneumoniae BAA 1705	bla_{KPC}		≥8	≥16	≥16
K.pneumoniae NCTC 13442	bla_{OXA-48}		≥8	≥16	≥16
Negativne kontrole					
E. coli ATCC 25922	-		≤ 0.125	≤ 0.25	≤ 0.25
E. coli ATCC 35218	bla_{TEM-1}		≤ 0.125	≤ 0.25	≤ 0.25

Table 2. Carbapenem susceptibility of carbapenemase-negative isolates.

Species name	Enzyme (phenotypic)	Number of isolates	Ertapenem MIC (µg/ml)	Imipenem MIC (µg/ml)	Meropenem MIC (µg/ml)
E. aerogenes	ESBL	1	0,5	2.0	0.5
S. marcescens	ESBL	1	≥8	0.5	1.0
S. marcescens	AmpC	1	0.5	1.0	0.25
P. mirabilis	ESBL	1	0.5	3.0	0.25

The RAPIDEC® CARBA NP test was used for colorimetric detection of carbapenemases. Out of 56 tested isolates, after the first reading (30 minutes), the test was positive for 32 carbapenemase - producing isolates, negative for 20 carbapenemase - producing isolates, and negative for four carbapenemase - negative isolates. The sensitivity and specificity of the test were 61.54% and 100.00%, respectively. After the second reading (120 minutes), the test was positive for 47 carbapenemase-producing isolates, negative for all four carbapenemase-negative isolates (no false-positive results), and false-negative for five isolates. Among the 20 false-negative isolates from the first reading (30 minutes), 15 isolates tested positive after 120 minutes: three NDM-positive *K. pneumoniae* isolates, ten OXA-48/NDM-positive *K. pneumoniae* isolates (mucoid strains), one OXA-48/NDM-positive *K. pneumoniae* isolate, and one NDM-positive *K. pneumoniae* isolate. The last two isolates had MIC values of ertapenem/meropenem/imipenem at 4/0.25/1 µg/mL and NDM 8/0.25/1 µg/mL (respectively). False-negative results were most commonly observed in OXA-48/NDM-positive mucoid *K. pneumoniae* isolates. Thus, after 120 minutes, the test's sensitivity and specificity increased to 90.38% and 100.00%, respectively. The results for sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the Carba NP test are presented in Table 3.

Table 3. Carba NP Test Results

Procedure	Number of isolates after 30 min.of incubation	Number of isolates after 120 min. of incubation
True-positive	32	47
True-negative	4	4
False-positive	0	0
False-negative	20	5
Sensitivity	61.54%	90.38%
Specificity	100.00%	100.00%
PPV	100.00%	100.00%
NPV	16.67%	44.44%

The results of the false-negative tests observed in five isolates after 120 minutes are presented in Table 4.

Table 4. Composition of the five carbapenemase-positive isolates that tested negative after 120 minutes.

No of isolates	Species name	Carbapenemase (PCR)	Result
3	*K. pneumoniae*	OXA 48	-
1	*K. pneumoniae*	OXA 48/NDM	-
1	*S. marcescens*	NDM	-

Carbapenemase production was evaluated using the CDT method with the commercial KPC/Metallo-beta-lactamase and OXA-48 Confirm Kit (Rosco Diagnostica). For the detection of carbapenemases using this test, simultaneous testing was performed using a meropenem disk along with disks containing meropenem/dipicolinic acid, meropenem/boronic acid, meropenem/cloxacillin, and temocillin. The combined disk test is interpreted based on the difference in the inhibition zone around the carbapenem disk and the disk containing both the carbapenem and the enzyme inhibitor. An increase in the inhibition zone around the combined carbapenem/inhibitor disk compared to the carbapenem disk alone, exceeding the defined threshold values, indicates a positive result.

For carbapenemase-positive bacteria that produce only NDM enzymes, the combined disk test using EDTA was negative in only 2 out of 24 isolates (one *M. morganii* isolate and one *K. pneumoniae* isolate) and positive in 22 out of 24 isolates. The combined disk test using DPA yielded the same results, with 2 negatives out of 24 isolates (one *E. cloacae* isolate and one *K. pneumoniae* isolate), and 22 out of 24 isolates testing positive. The combined disk test with boronic acid was positive in eight OXA-48-positive isolates and two NDM-positive isolates. Among the OXA-48-positive isolates, none produced a positive result with either the CDT-BA and CDT DPA tests, and all were resistant to temocillin. Both CDT cloxacillin and the boronic acid test were positive in only one NDM isolate, indicating co-production of AmpC.

The combined disk test using DPA was positive in 7 out of 12 isolates, while the EDTA-based test was positive in 6 out of 12 isolates. The combined disk test with boronic acid was negative in both KPC-positive isolates. According to the manufacturer's instructions, the temocillin test could only be interpreted as positive in three OXA-48/NDM-positive *K. pneumoniae* isolates; for the other isolates, interpretation was not possible due to the positive results in the DPA and boronic acid tests. The combined disk test with cloxacillin was negative for all tested isolates.

The combined disk tests using DPA/EDTA and the temocillin test were negative for all carbapenemase-negative isolates. The CDT test with boronic acid and cloxacillin, which serve as indicators of AmpC enzyme production, were positive in one *Serratia marcescens* isolate, which was also identified by Vitek 2 AES as a potential AmpC producer.

The NG-Test CARBA 5 was positive in all isolates (*K. pneumoniae* isolate) for carbapenemase-positive bacteria that produce only NDM and OXA 48 enzymes.

For carbapenemase-positive bacteria that produce multiple enzyme types NG-Test CARBA 5 was negative in only 1 out of 12 isolates (two *K. pneumoniae* isolates) and positive in 11 out of 12 isolates. In *K. pneumoniae* that produced all three enzymes, the test detected only the NDM enzyme.

The results for sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are presented in Table 5.

Table 5. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of RAPIDEC Carba NP (RCNP), NG-Test CARBA 5, Rosco Combined Disk Test for Carbapenemase Production, Combined Disk Test

Phenotypic test	Detection of carbapenemase	Sensitivity	Specificity	PPV	NPV
RCNP[1] after 120 min.	Carbapenemase	90.38%	100.00%	100.00%	44.44%
NG-Test CARBA 5	KPC, NDM, OXA-48-like	98.08%	100.00%	100.00%	80.00%
RCDT[2] Temocilin	OXA-48	95.65%	100.00 %	100.00%	83.33 %
RCDT[2] Boronic acid	Class A	0.00%	77.78 %	0.00%	95.45 %
RCDT[2] DPA	Class B (MBL)	80.56%	100.00 %	100.00%	74.07
CDT[3] EDTA	Class B (MBL)	80.56%	100.00 %	100.00%	74.07

[1]RAPIDEC Carba NP [2] Rosco Combined Disk Test for Carbapenemase Production [3]Combined Disk Test

The technical characteristics of the tests, including complexity and execution time, the number of steps required, time to result, and whether the test identifies specific carbapenemase classes or only detects their presence, are presented in Table 6.

Table 6. The technical characteristics of the phenotypic tests

Characteristics	Phenotypic test		
	CDT	RCNP	NG-Test Carba 5
Validated isolate set	*Enterobacterales*		
	KPC, Class B (MBL), OXA-48-like	Carbapenemase	KPC, NDM, OXA-48-like
Maximum incubation time (hours)-Time to result readout	18-24	2	0.25
Total operator time (minutes)	5	5	3
Number of steps required	4	3	3
Operational simplicity	Easy, training required	Easy, little training required	Very easy, no training required

DISCUSSION

Global dissemination of carbapenemase-producing *Enterobacterales* necessitates rapid and efficient detection methods in routine laboratory work (11), (12). Detecting and monitoring these strains in hospital settings is essential not only for controlling hospital-acquired infections but also for tailoring individual therapeutic strategies for infections caused by carbapenemase-positive strains (13). The emergence of CRE requires that all *Enterobacterales* isolates exhibiting reduced susceptibility to one or more carbapenems be tested using efficient methods available in routine practice.

Rapid detection of carbapenemase production in *Enterobacterales* is crucial for preventing the spread of these strains, especially in hospital settings. Although PCR is regarded as the reference method for detecting carbapenemases, many authors report phenotypic methods as reliable and accessible means of detecting these enzymes.

A wide range of tests for detecting the production of carbapenemases is used in both routine diagnostics and epidemiological studies. In this research, isolates that are potentially carbapenemase producers—considered the most clinically and epidemiologically significant—were selected based on the threshold values provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (9). After selection, we used three phenotypic methods for the detection of carbapenemases and compared them with Multiplex PCR.

Detection of carbapenemases using the combined disk test (CDT), has been reported by many authors to provide satisfactory results, considering their sensitivity and specificity (14), (15), (16).

In our study, the RCDT demonstrated good performance in detecting MBL enzymes, with a sensitivity and specificity of 80.56%/100%, and PPV/NPV of 100%/74.07% when using DPA as the inhibitor. Identical results were obtained with the CDT using EDTA as the inhibitor. Solgi et al. reported sensitivity and specificity values of 82.61% and 96.22% for the CDT DPA test (17). These findings are consistent with previous reports (14).

Regarding the detection of KPC enzymes, the results showed low values.The combined disk test with boronic acid was positive in eight OXA-48-positive isolates and two NDM-positive isolates but in both KPC producers test was negative. Sensitivity and specificity were 0%/77.78%, and PPV/NPV of 0%/95.45%. It should be noted that the number of isolates evaluated was small (2), and both isolates were co-producers of NDM or OXA-48 enzymes. Certainly, the small number of KPC-producing organisms in our study limits our ability to draw robust conclusions from the data. Our findings do not align with those of Dijk et al (7), who demonstrated that the PBA test effectively detects carbapenemase production in CRE isolates, with sensitivity and specificity rates of 95% and 99%, respectively.

Our sensitivity and specificity results indicate the good performance of temocillin in detecting OXA-48 enzymes (95.65%/100%). It is crucial to note that the inhibition zone around the temocillin disk should only be considered valid when there is no difference in the inhibition zones between meropenem alone and meropenem combined with class A and MBL inhibitors. The sensitivity of this method has also been confirmed by other authors (18). The RCDT, as currently designed, has been assessed in multiple studies, demonstrating high sensitivity ranging from 90% to 100%, depending on the carbapenemase type, and a specificity of 92% to 93% (7), (19). Bartolini et al. reported 100% sensitivity and 100% specificity for RCDT (20). However, this author, along with others (21), (22), highlights challenges in detecting isolates that produce multiple types of carbapenemases. In our study, the combined disk test using DPA was positive in 7 out of 12 isolates, while the EDTA-based test was positive in 6 out of 12 isolates.

The sensitivity and specificity of the RAPIDEC CARBA NP (RCNP) test in our study increased to 90.38% and 100.00%, PPV 100% and NPV 44,44%) after 120 minutes, compared to the readings at 30 minutes. Noel et al. reported a sensitivity/specificity of 91.9%/83.9% for the RCNP test (23). In Alizadeh et al study, the sensitivity and specificity of Carba NP test was 98% and 95%, respectively (24). In newer research, RCNP test showed an overall sensitivity, specificity, PPV, NPV and accuracy of 69.3%, 100%, 100%, 6.9%, respectively (25). Our research indicated that false-negative isolates were most frequently observed in OXA-48/NDM-positive mucoid isolates of *K. pneumoniae*. Similar

findings, particularly regarding the detection of OXA-48 producers, have been reported in other studies (26), (27), (28).

In our study, sensitivity, specificity, PPV, and NPV for NG-Test CARBA 5 were 98.08%/100.00% and 100.00%/80.00%, respectively. In another study (Hopkins et al), the overall sensitivity and specificity of the NG-Test CARBA 5 were 97.31% (95% CI 93.84%–99.12%) and 99.75% (95% CI 99.12%–99.97%), respectively (29). Saito et al. state that the NG-Test CARBA 5 demonstrated a sensitivity of 99.1% (106 out of 107 strains of the five most common carbapenemase producers) and a specificity of 100% for *Enterobacterales* strains (30). The same study shows false-negative results for IMP producers, which has also been reported by other authors (31).

Regarding the strains with multiple carbapenemase genes, NG-test Carba 5 successfully identified all these carbapenemases. However, the sensitivity and specificity of each method varied for different kinds of carbapenemases.

All three tests significantly reduced the turnaround time to under two hours, enabling direct identification of carbapenemases from clinical samples. For *Klebsiella spp*., the accuracy of the Carba 5 test was 96.82% (5).

Study Limitations

The main limitation of this study is the relatively small number of isolates in which carbapenemases were detected using the reference PCR method. However, the Center for Microbiology regularly monitors the occurrence of carbapenemases using phenotypic methods. The types and distribution of specific carbapenemases correspond to the group of isolates defined by molecular methods. More extensive research, including a larger number of isolates and both genotypic and phenotypic methods for carbapenemase detection, is necessary.

Conclusion

It is clear that the detection and differentiation of carbapenemases are no longer solely important for epidemiological surveillance and infection control but play a crucial role in selecting appropriate therapy and implementing antimicrobial stewardship strategies, especially considering the availability of novel antimicrobial agents targeting specific carbapenemases.

This study demonstrated that, based on their performance, available phenotypic tests can serve as useful methods for detecting carbapenemases in carbapenem-resistant *Enterobacterales* in routine practice. Although the RCNP test is simple to perform and provides results within two hours, it does not differentiate between different types of carbapenemases. Since susceptibility to newer

antimicrobial agents is directly related to the enzyme type, it is essential for phenotypic methods to identify the specific carbapenemase present.

For this reason, the RCDT method represents a relatively inexpensive option that requires neither specialized training nor equipment, offers good performance, and can classify different carbapenemase classes. However, its main drawback is the longer turnaround time of 18–24 hours. This method is suitable for epidemiological surveillance and use during hospital outbreaks.

Finally, the NG-Test CARBA 5 demonstrated excellent accuracy in detecting carbapenemase-producing strains, with high sensitivity and specificity. The test is extremely simple, requires no special equipment or personnel training, and differentiates between five different carbapenemase types.

Phenotypic methods have limitations related to the types of carbapenemases they can detect, the challenge of identifying strains that produce multiple carbapenemases, and the varying distribution of specific enzymes across different geographical regions. Perhaps the most effective approach in selecting an appropriate test would be to conduct molecular screening in a given region to determine the presence of specific carbapenemases and then choose the most reliable test for those predominant enzymes.

Based on our molecular data indicating the predominance of NDM and OXA-48 enzymes, RCDT would be a suitable method for epidemiological studies, whereas the NG-Test CARBA 5 would be ideal for the rapid detection of carbapenemase producers, particularly in clinical samples requiring urgent processing (e.g., blood cultures, cerebrospinal fluid). An important future development would be refining this test to enable the direct detection of carbapenemases from patient samples (e.g., blood, urine).

Ultimately, continuous surveillance of the presence and spread of carbapenemases in hospital environments is essential for epidemiological monitoring, infection control, and the implementation of effective antimicrobial therapy.

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