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Emerging Problems in Infectious Diseases

A comparison of phenotypic methods for the detection of carbapenemase in *Klebsiella pneumoniae*

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Abstract

Introduction: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates are increasingly spreading in the community and pose a serious public health threat. Rapid and accurate detection of these isolates is therefore critical. This study aimed to evaluate the performance of combined disc method, the carbapenem inactivation method (mCIM) and its modifications.

Methodology: A total of 100 *K. pneumoniae* isolates collected from various clinical samples between 2018 and 2021 were included. Carbapenemase production was investigated using the combined disc method, mCIM (19 mm and 22mm), and aCIM (19 mm and 22 mm). Carbapenemase types were determined using the combined disc method, eCIM, and mCIMplus. Resistance genes were detected by real-time PCR, which was used as the reference method to evaluate the performance of phenotypic tests.

Results: For carbapenemase detection, the sensitivities of combined disc method, mCIM-19 mm, mCIM-22 mm, aCIM-19 mm, and aCIM-22 mm were 100%, 84.8%, 95.7%, 83.7% and 95.7%, respectively, while the specificities are 62.5%, 100%, 25%, 100%, and 62.5%. For carbapenemase species detection, the sensitivities of the combined disc method, eCIM-19 mm, eCIM-22 mm, and mCIMplus were 98,9%, 75.4%, 84% and 26.1%, respectively; the specificities were 62.5%, 48.8%, 42% and 100%. No significant differences were observed between the mCIM and aCIM tests. (*p* value:1 for 19 mm, *p* value:0.37 for 22 mm).

Conclusions: The combined disc method provided the most effective balance of performance and practicality. However, phenotypic methods may be insufficient for isolates harboring multiple resistance genes.

Key words: Carbapenem-resistant *Klebsiella pneumoniae*; carbapenemase; combined disc method; carbapenem inactivation method.

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Introduction

The members of the family *Enterobacterales* can be pathogenic in many clinical cases, ranging from simple community-acquired infections to medically care-associated infections. An increase in antimicrobial drug resistance in infections has caused an increase in morbidity and mortality rates. Carbapenems are generally regarded as last-resort antibiotics for the treatment of *Enterobacterales* infections, particularly those caused by isolates producing extended-spectrum β-lactamases (ESBLs) and exhibiting resistance to multiple other antibiotics. However, the increasing prevalence of carbapenem resistance has resulted in limited treatment options and higher mortality rates [1,2]. Carbapenem resistance is primarily mediated by the production of carbapenemase enzymes which are classified into class A (i.e., KPC and GES), class B (i.e., VIM, NDM, and IMP), and class D (OXA-48-like) [3]. These resistant genes can quickly transmit among bacteria via mainly plasmids and spread rapidly [4]. To select the appropriate antibiotic for treating carbapenem-resistant *Enterobacterales* infections, it is important to identify the type of carbapenemase gene (e.g., KPC, NDM, OXA-48) and its class (e.g., A, B, D). For example, ceftazidime-avibactam is ineffective against Class B carbapenemases, while meropenem-vaborbactam is only effective against Class A carbapenemases [5]. Therefore, quick and accurate detection is of great importance. The molecular methods that are used as a gold standard in detecting these resistances have a high specificity and sensitivity [6]. However, its use is not as widespread as desired, as it is an expensive method requiring experienced personnel. Access to molecular methods is limited in many laboratories, particularly in developing countries. Various phenotypic methods have been developed for this purpose.

Carbapenem inactivation method (CIM) is a simple and low-cost method to detect the presence of carbapenemase and give results quickly [7]. CLSI has updated the CIM method in 2017 to the modified carbapenem inactivation method (mCIM) by adding Tryptic Soy Broth (TSB) instead of distilled water and increasing the incubation time of the isolates tested on meropenem disc from 2 to 4 hours [8]. To differentiate metallo-β-lactamase (MBL) enzymes from other

carbapenemases, the EDTA-supplemented carbapenem inactivation method (eCIM), performed by adding EDTA to TSB according to CLSI 2018, was incorporated [9]. Petit *et al.* have added EDTA and phenylboronic acid (PBA) to TSB to develop the modified carbapenem inactivation method plus (mCIMplus) method used for distinguishing Class A, B, and D carbapenemases [10]. Gelmez *et al.* developed the mCIM test and studied the modified carbapenem inactivation method with ammonium bicarbonate (aCIM). In their study, they evaluated the aCIM test according to the EUCAST guideline meropenem breakpoint value of 22 mm, in addition to the 19 mm breakpoint value recommended in CLSI 2018. They have stated that OXA-48 and NDM-producing isolates were better detected with these modifications [11]. Studies have indicated that the performance of various phenotypic tests differs in isolates containing KPC, NDM, and OXA-48 enzymes [11-13].

Due to the increasing incidence of CRE, dedicated surveillance networks have been established for their monitoring. While the ertapenem and imipenem/meropenem resistances were 9% and 3% respectively for *Escherichia coli* according to the Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR) 2020 report, these values were 51% and 39%, respectively for *Klebsiella pneumoniae* [14]. According to a study by Rodríguez-Baño *et al*., *K. pneumoniae* is the *Enterobacterales* with the highest frequency of carbapenemase production [15].

Given the rising resistance rate of *K. pneumoniae*, this study aimed to evaluate the sensitivity and specificity of several phenotypic methods for detecting carbapenemases. We analyzed 100 *K. pneumoniae* isolates representing CRE using mCIM and ammonium bicarbonate modified mCIM (aCIM) for enzyme detection, and eCIM and mCIMplus for enzyme differentiation. Results were assessed using both 19 mm and 22 mm inhibition zone thresholds. Additionally, the combined disc method was used for both detection and differentiation, and results were compared with molecular reference methods. This study aimed to evaluate and compare the performance of combined disc, mCIM, aCIM, eCIM, and mCIMplus tests in detecting and classifying carbapenemase types in clinical *K. pneumoniae* isolates.

Methodology

Bacterial Isolate Selection

Our study included 100 *K. pneumoniae* isolates obtained from various clinical specimens, including urine, sputum, tracheal aspirate, and bronchoalveolar lavage, submitted to the Uşak Medical Microbiology Laboratory from inpatient wards and intensive care units between 2018 and 2021. Another inclusion criterion for these isolates was their compliance with the EUCAST carbapenemase screening guidelines. Isolates that did not meet these criteria were excluded from the study. The identification of these isolates and antibiotic susceptibility testing were performed in the laboratory using automated methods with GN, AST 423 and AST 420 cassettes (Vitek 2 Compact 60, bioMerieux, France).

For patients with recurrent isolation of the same pathogen, only one isolate was included in the study. Isolates were stored in stock medium (Mast Cryobank®, Mast Group Ltd, England) at -80ºC until the day of the experiment.

Combined Disc Method

Phenotypic detection of KPC, MBL, and OXA-48 carbapenemases in bacterial isolates was performed using the KPC, MBL, and OXA-48 combined disc kit (Liofilchem®, Italy). The test was carried out based on the manufacturer's recommendations. The evaluation was conducted by observing the increase in the zone diameter of discs containing the inhibitor and according to EUCAST's comments and the manufacturer's recommendations.

Modified Carbapenem Inactivation Method (mCIM)

To obtain a pure culture, the day before the study, isolates were subcultured from stock media onto 5% sheep blood agar (RTA Lab, Turkey). 1 µL of the pure culture colony from the bacteria to be tested was added to 2 mL of Tryptic Soy Broth (TSB) medium (Oxoid, Thermo Scientific, USA). 10 µg meropenem disc (Oxoid, Thermo Scientific, USA) was added to the bacterial suspension and incubated in an incubator at 35°C for 4 hours. Following incubation, the meropenem disc was placed in MHA medium (RTA Lab, Türkiye) containing a spread of carbapenem sensitive *E. coli* ATCC® 25922, followed by an incubation in an incubator at 35°C for an additional 16-20 hours. Interpretation followed CLSI guidelines [9].

Ammonium bicarbonate modified carbapenem inactivation method (aCIM)

The procedure is the same as in the mCIM method. Additionally, ammonium bicarbonate (Sigma A6141) (pH:7.0) was adjusted to a pH of 7.0 at the final concentration of 50mM in the bacterial suspension. Evaluation of incubated plates was carried out in two different ways in compliance with the evaluation

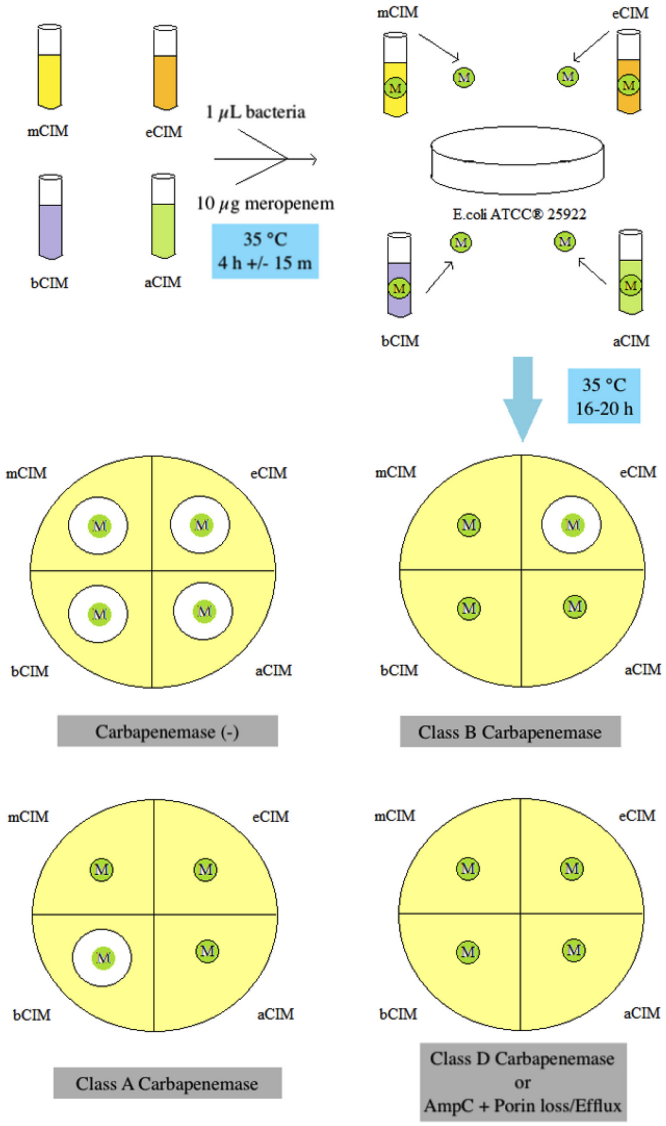
criteria in CLSI 2018 and the study by Gelmez *et al.* [11].

EDTA modified carbapenem inactivation method (eCIM)

This method used two tubes: one containing 2 mL of Tryptic Soy Broth (TSB) alone, and the other containing 2 mL of TSB supplemented with EDTA to a final concentration of 5 mM (20 µL of 0.5 M EDTA). 1 µL of the pure culture colony from the bacteria to be tested was added to both tubes. The subsequent steps were carried out as in the mCIM test. Following incubation, interpretation followed CLSI guidelines [9].

Phenylboronic acid (PBA) modified carbapenem inactivation method (bCIM)

Figure 1. Study and evaluation of modified carbapenem inactivation methods (Prepared using the table in Tsai et al. [20].



Two tubes were used for this method, one containing 2 mL Tryptic Soy Broth (TSB) medium, and the other containing 0.07 mL of PBA solution (final concentration of 20 mg/mL) obtained by dissolving 40 mg of phenylboronic acid (Glentham GK1609) in 2 mL of dimethylsulphonic acid (DMSO) added to 2 mL of TSB medium. 1 µL of the pure culture colony from the bacteria to be tested was added to both tubes. The subsequent steps were carried out as in the mCIM test. Following incubation, interpretation of the mCIMplus test (combining mCIM, eCIM, and bCIM) followed the algorithm described by Petit *et al.* [10].

In the mCIMplus test evaluation, isolates showing an inhibition zone of 10 mm or less on the meropenem + EDTA disc and an inhibition zone greater than 10 mm on the meropenem + PBA disc are classified as Class A; isolates showing an inhibition zone greater than 10 mm on the meropenem + EDTA disc and an inhibition zone of 10 mm or less on the meropenem + PBA disc are classified as Class B; isolates showing an inhibition zone of 10 mm or less on both discs are classified as class D or 2. Isolate results showing an inhibition zone greater than 10 mm on both discs are classified as 'unreliable'.

The steps for the procedure and evaluation of the modified carbapenem inactivation methods are shown in Figure 1. The zone diameters in the plaques were evaluated separately based on the interpretation criteria of mCIM, aCIM, eCIM, and mCIMplus tests (Figure 2).

Figure 2. Interpretation of mCIM, acIM, eCIM and mCIMplus tests for isolates A, B and C.

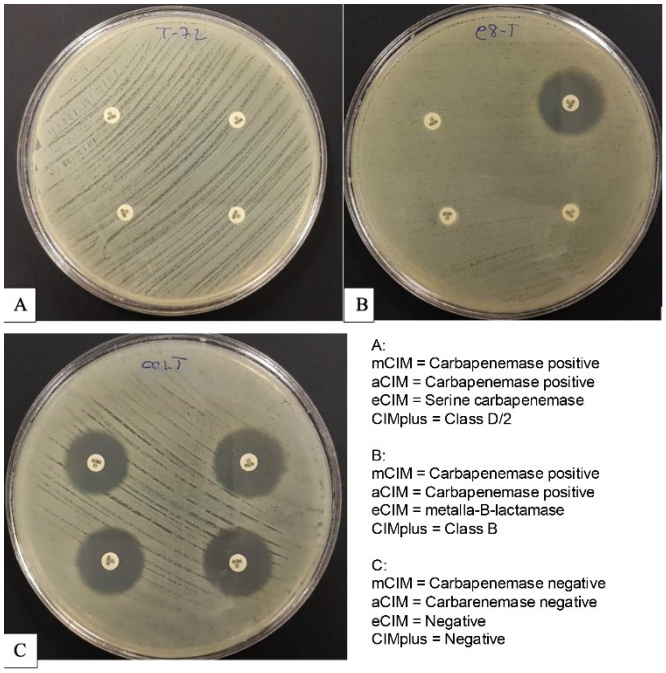


Table 1. Genotypic analysis results of carbapenemase genes of isolates.

Carbapenemase	NDM + OXA-48	OXA-48	KPC	NDM	VIM + OXA-48	KPC + OXA-48	VIM	Negative	Total
Number (n)	40	34	6	5	4	3	1	8	100
Ratio (%)	40	34	6	5	4	3	1	8	100

Carbapenemase Gene Detection

Real-time PCR method (Carbapenem Resistance qPCR Kit, Bioeksen®, Türkiye) was used in our study to identify the bla*KPC*, bla*NDM*, bla*VIM*, bla*IMP*, bla*OXA-51*, bla*OXA-23*, bla*OXA-58*, and bla*OXA-48* genes. Firstly, Rapid Nucleic Acid Extraction Kit (Bioeksen®, Türkiye) was used with Zybio EXM 3000 for extracting nucleic acids from bacteria using the magnetic bead method. DNA extraction and Real-time PCR method were performed according to the manufacturer's recommendations.

Statistical analysis

SPSS for Windows version 20.0 package program was used for statistical analysis. Cross-tabulations were used to determine the relationships between two variables. PCR results were taken as the gold standard, while the sensitivity and specificity of the combined disc method, mCIM, eCIM, bCIM, aCIM in detecting the carbapenemase were calculated. The McNemar test was performed to calculate the effect of variables in the mCIM and aCIM tests. Statistical significance was set at $p < 0.05$.

Results

One hundred *K. pneumoniae* isolates that met the carbapenemase screening criteria defined by the EUCAST 2021 guidelines were included in the study. At least one carbapenemase enzyme was detected by PCR in 92 of these isolates, while the enzymes in the kit used were found to be negative in 8 of them. The genotypic carbapenemase results of the isolates according to PCR method are given in Table 1.

Among the phenotypic methods evaluated for the detection of carbapenemase production, the combined disc method, mCIM, and aCIM assays were performed and compared in terms of sensitivity and specificity. The mCIM and aCIM tests were assessed using two different cutoff thresholds (19 mm and 22 mm). The performance characteristics of the phenotypic tests for carbapenemase detection are summarized in Table 2.

The combined disc method, eCIM, and mCIMplus (a combination of mCIM, eCIM, and bCIM) methods were used to determine carbapenemase types. The eCIM test was evaluated using two different cutoff thresholds, 19 mm and 22 mm. The evaluation results of the tests performed for carbapenemase type identification are presented in Table 3.

When the eCIM test was evaluated using the 19 mm cutoff threshold, five of the 34 OXA-48-producing isolates were correctly identified and categorized as a serine carbapenemase. However, 22 of them were included in class B resulting false positives, and seven of them gave false negatives after being detected as carbapenemase negative. When the cutoff threshold value was set at 22mm, the seven isolates testing negative for carbapenemase in 19 mm were categorized into the serine carbapenemase class accurately.

In the mCIMplus test, all KPC, KPC + OXA-48, VIM, and VIM + OXA-48 isolates were correctly classified. Of the NDM isolates, two were correctly classified, while three were detected as negative. One NDM + OXA-48 isolate was classified as class A, 18 were classified as negative, and 14 were classified as 'unreliable' and thus considered 'false negatives'. Four OXA-48 isolates were classified as class A or B, 11 were classified as negative, and 17 were classified as 'unreliable' and thus considered 'false negatives'.

Discussion

The results obtained from phenotypic and molecular comparisons were interpreted in comparison of previous studies. We evaluated the performance of the combined disc method, mCIM and aCIM tests to detect carbapenemase production. In our study, the sensitivity of the combined disc method was 100%, similar to previous studies [16,17]. However, the

Table 2. Comparison of the results of combined disc method, mCIM (19 mm), mCIM (22 mm), aCIM (19 mm), aCIM (22 mm) tests used to detect the presence of carbapenemases.

Presence of carbapenemase	Combined disc method	mCIM		aCIM	
		< 19 mm	< 22 mm	< 19 mm	< 22 mm
True Positive	92	78	88	77	88
False Positive	3	0	6	0	3
True Negative	5	8	2	8	5
False Negative	-	14	4	15	4
Sensitivity (%)	100	84.8	95.7	83.7	95.7
Specificity (%)	62.5	100	25	100	62.5

Table 3. Comparison of the results of the combined disc method, eCIM (19 mm), eCIM (22 mm), and mCIMplus tests used to detect the type of carbapenemase.

Carbapenemase	Combined Disc Method						eCIM (19 mm)						eCIM (22 mm)						mCIMplus					
	TP	FN	TN	FP	Sv	Sp	TP	FN	TN	FP	Sv	Sp	TP	FN	TN	FP	Sv	Sp	TP	FN	TN	FP	Sv	Sp
NDM +OXA-48	40	0	-	-	100	-	34	6	-	-	85	-	33	7	-	-	82.5	-	7	33	-	-	17.5	-
OXA-48	33	1	-	-	97.1	-	-	7	5	22	-	18.5	-	-	12	22	-	35.3	2	32	-	-	5.9	-
KPC	6	0	-	-	100	-	-	-	6	0	-	100	-	-	6	0	-	100	6	0	-	-	100	-
NDM	5	0	-	-	100	-	4	1	-	-	90	-	4	1	-	-	90	-	2	3	-	-	40	-
VIM +OXA-48	4	0	-	-	100	-	4	0	-	-	100	-	4	0	-	-	100	-	4	0	-	-	100	-
KPC +OXA-48	2	0	-	-	100	-	-	-	2	0	-	100	-	-	2	0	-	100	2	0	-	-	100	-
VIM	1	0	-	-	100	-	1	0	-	-	100	-	1	0	-	-	100	-	1	0	-	-	100	-
Negative	-	-	5	3	-	71.4	-	-	8	0	-	100	-	-	1	7	-	12.5	-	-	8	0	-	100
Total	91	1	5	3	98.9	62.5	43	14	21	22	75.4	48.8	42	8	21	29	84	42	24	68	8	0	26.1	100

TP: True positive, FN: False negative, TN: True negative, FP: False positive, Sv: Sensitivity, Sp: Specificity.

specificity was 62.5%, which was lower than that reported in those studies. The specificity of the mCIM test in our study was comparable to that reported in the CLSI 2018 guidelines [9]. However, the sensitivity in our study (84.8%) was lower, similar to Gelmez *et al.* (81.95%) [11]. Tsai *et al.* similarly found the sensitivity of the mCIM test conducted on 195 *Enterobacterales* isolates as 89.3% and the specificity as 98.7% [18].

Previous studies have demonstrated that bicarbonate enhances the activity of carboxylated lysine residues in OXA-type enzymes' active site. The addition of bicarbonate in assays based on meropenem hydrolysis has been shown to facilitate the detection of OXA-type carbapenemases [19,20]. In our study, the sensitivity remained almost the same after ammonium bicarbonate addition (83.7%), whereas the specificity was determined to be 100%. One additional OXA-48-producing isolate was detected by aCIM that had not been identified by mCIM; however, both NDM-producing isolates yielded false-negative results.

In our study, both the mCIM and aCIM tests were evaluated using cutoff thresholds of 19 mm and 22 mm. When the cutoff value was set at < 22 mm, the sensitivity of both tests increased; however, specificity decreased due to an increase in false-positive results. The addition of ammonium bicarbonate did not result in a statistically significant difference in the mCIM performance at either threshold ($p = 1$ for 19 mm, $p = 0.37$ for 22 mm). Among the methods evaluated for the detection of carbapenemase production, the combined disc method demonstrated the highest sensitivity. The performance of the aCIM test using the 22 mm cutoff threshold yielded sensitivity and specificity values comparable to those of the combined disc method.

We further compared the combined disc method, eCIM and mCIMplus tests for the identification of carbapenemase types. Giske *et al.* showed that KPC, OXA-48, and ESBL isolates gave a false positive in the combined disc method [21]. Similarly, in our study, three of the eight carbapenemase-negative isolates yielded false-positive results with the combined disc method: two were misidentified as OXA-48 producers and one as a KPC producer.

Previous studies have compared the mCIM and aCIM tests for the detection of carbapenemase production. Several emonstrated that the presence of OXA-48 and NDM enzymes—particularly when co-produced—may complicate phenotypic detection and lead to reduced sensitivity and false-negative results [11,22,23]. Similarly, in our study, fourteen isolates were classified as false negatives by the mCIM test. Of these, thirteen harbored the OXA-48 enzyme, and six of them additionally carried the NDM enzyme. When the mCIM cutoff threshold was increased from < 19 mm to < 22 mm, five NDM + OXA-48 co-producers, four OXA-48 producers, and one NDM producer that had not been detected at the < 19 mm threshold were identified. Consequently, the sensitivity increased from 84.8% to 95.7%. However, increasing the cutoff threshold resulted in false-positive results in six of the eight carbapenemase-negative isolates, leading to a marked decrease in specificity from 100% to 25%.

When the cutoff threshold for the aCIM test was set at < 22 mm, it was possible to detect the four NDM + OXA-48, four OXA-48, and three NDM isolates that were not detected in the < 19 mm cutoff threshold value. The test's sensitivity increased from 83.7% to 95.7% in this case. Three of the eight isolates not containing carbapenemase gave a false positive when the cutoff threshold value was increased. Hence, the specificity decreased from 100% to 62.5%.

The eCIM method was suggested to differentiate metallo-β-lactamases from serine carbapenemases in the CLSI 2018. Sensitivity was stated as > 95% and specificity as > 92% in various studies. Tsai *et al.* further noted that the false negative results contributing to reduced sensitivity were associated with isolates harboring IMP and NDM + OXA-48 enzymes' gene regions. Fourteen isolates were evaluated as false negatives in the eCIM test. Thirteen of these carried the

OXA-48 enzyme, and six additionally harbored the NDM enzyme. Tsai *et al.* also reported that false positive results leading to reduced specificity were associared with isolates containing the OXA enzyme gene region. Similarly, the OXA enzyme gene region is present in all isolates, giving false positive results in our study.

Unlike previous studies, we evaluated and compared the eCIM test using two different cutoff thresholds: 19 mm and 22 mm. A slight increase in sensitivity was observed when the 22 mm cutoff was applied compared with the 19 mm threshold, as six OXA-48-producing isolates that had been classified as false negatives at the 19 mm cutoff were reclassified as true negatives at 22 mm. Although the 22 mm threshold improved the detection of OXA-type enzymes in the eCIM, mCIM, and aCIM assays, the associated increase in false-positive results led to reduced specificity, representing a major limitation of this approach.

Petit *et al.* evaluated the mCIMplus test by adding EDTA and PBA to mCIM test in a study including 159 CRE isolates. They reported the sensitivity of the test as 98.5% and the specificity as 100% [10]. In contrast to the other two studies, the sensitivity of our study was lower at 26.1%, while its specificity was the same at 100%. According to the test results, the highest rate of false-negative results was associated with OXA-48 and NDM + OXA-48 isolates. Detailed analysis of the false-negative results revealed that 31 isolates were categorised as 'unreliable' according to the evaluation algorithm, as they produced inhibition zones > 10 mm inhibition zones on both EDTA and PBA discs. We believe that the classification of nearly one-third of the isolates as unreliable contributed substantially to the reduced in sensitivity. Furthermore, 80% of the isolates in our study harbored the OXA-48 enzyme. The lack of a specific inhibitor targeting OXA-48 enzyme may have contributed to the lower-than-expected sensitivity. Furthermore, in our study, only six of the 55 isolates containing dual-resistant enzymes were classified as 'Class D/2'. We believe that the use of this test is not appropriate in regions where isolates containing the OXA-48 enzyme and dual-resistant enzymes are predominant.

When comparing the tests we used to identify carbapenemase types, the combined disc test demonstrated the highest sensitive. In the eCIM test, false-positive results -particularly in the OXA-48 enzyme- reduced specificity. Although the mCIMplus test exhibited 100% specificity, its sensitivity was low, largely because OXA-48-producing isolates were frequently classified as false negatives. We believe that, in regions where OXA-type enzymes are endemic, the selection of a diagnostic method should be guided by these performance characteristics.

Study limitations

One limitation of our study is that bacterial isolates belonging to other genera within the *Enterobacterales* order were not be included in the assessment of carbapenemase detection methods. Another limitation is the limited number of Klebsiella pneumoniae isolates producing class A carbapenemases in our sample set, which precluded a reliable calculation of specificity for this subgroup.

Conclusions

In conclusion, based on the sensitivity and specificity of the phenotypic assays evaluated in this study, the combined disc test demonstrated the highest diagnostic reliability for detecting carbapenemase production, particularly among OXA-type producers. However, further studies are needed to expand current knowledge on carbapenem inactivation methods. Future investigations should include a broader spectrum of *Enterobacterales* species harboring diverse carbapenemase types and should incorporate advanced techniques such as MALDI-TOF MS for comprehensive characterisation. It should be emphasized that phenotypic methods may be insufficient for the detection of isolates harboring multiple resistance genes. Therefore, until more reliable phenotypic tests are developed, the use molecular methods to determine carbapenemase type following phenotypic confirmation of carbapenemase production may be considered in routine clinical practice.

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Ethical statement

The study protocol was approved by the Ethics Committee of Tekirdağ Namık Kemal University Non-Interventional Clinical Research Ethics Committee with the decision dated 04.05.2021 and the number E-46048792-050.01.04-34577.

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Conflict of interest
No conflict of interest is declared.

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