

ORIGINAL ARTICLE

Comparative Study of Carbapenemase Inhibitor and NG-Test CARBA5 for the Detection of Carbapenemase of *Enterobacteriaceae*

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SUMMARY

Background: This study aimed to evaluate the application of carbapenemase inhibitor and NG-test CARBA5 in the detection of carbapenemase genotype for *Enterobacteriaceae*.

Methods: We collected non-repetitive CRE (carbapenem-resistant Enterobacterales) isolated from patients at the Affiliated Cancer Hospital of Zhengzhou University in 2022. The carbapenemase type and gene type were detected by carbapenemase inhibitor test and NG-test CARBA5 assay, respectively.

Results: Sensitivity and specificity of the carbapenemase inhibitor test detecting five common carbapenemases were 100.00% and 98.36%, respectively. Sensitivity and specificity of NG-test CARBA5 were 95.90% and 99.18%, respectively.

Conclusions: The carbapenemase inhibitor test and NG-test CARBA5 showed high sensitivity and specificity in detecting carbapenemase of *Enterobacteriaceae*.

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KEYWORDS

carbapenemase inhibitor test, NG-test CARBA5, carbapenem-resistant *Enterobacteriaceae* (CRE)

INTRODUCTION

Infection caused by carbapenem-resistant *Enterobacteriaceae* (CRE) is severe and complex, with a high incidence rate and mortality, and it has been listed as a high priority pathogen by WHO [1]. The “China CRE Network” showed that Henan Province has the highest detection rate of CRE across the country, with over 89% of CRE strains producing carbapenemase. The resistance mechanism of a few non-carbapenemase-producing CRE strains was the production of extended spectrum β -lactase or AmpC combined with downregulation of outer membrane protein expression [2]. The treatment of different types of carbapenemase strains is also different; rapid and accurate identification of carbapenemase types is of great significance for anti-infection and prevention of hospital-acquired infections. A-

class carbapenemase-producing strains are usually sensitive to polymyxin, cefotaxime/avibactam, tigecycline, and meropenem/vedobactam [3]. B-class carbapenemase-producing strains are usually sensitive to polymyxin, tigecycline, and azuron/avibactam.

There are two common methods for detecting carbapenemase: phenotype testing and genotype testing. The recommended phenotype detection methods in the guide include mCIM assay, Carpa NP assay, eCIM assay, carbapenemase inhibitor assay, etc. The commonly-used genotype detection methods in clinical practice testing include NG-test CARBA5 test, PCR, and molecular detection techniques. PCR, carbapenemase inhibitor test, and NG-test CARBA5 are highly recommended. There is little research on the application value and comparison of carbapenemase inhibitor and NG-test CARBA5 in detecting carbapenemase. Our study aimed to evaluate the clinical application value of carbapenemase inhibitor and NG-test CARBA5 in detecting carbapenemase.

MATERIALS AND METHODS

Clinical specimens

All of the 124 nonrepetitive CRE strains detected throughout the year were collected from hospitalized patients at the Affiliated Cancer Hospital of Zhengzhou University in 2022 and were stored at -80°C in 30% glycerol. The Affiliated Cancer Hospital of Zhengzhou University, the national regional cancer center, is the only tertiary cancer hospital in Henan province that integrates medical treatment, prevention, scientific research, teaching, and rehabilitation. The hospital has nearly 3,000 beds and more than 5,000 employees, with an annual outpatient volume of about 500,000 and an annual discharge of about 50,000. As Henan Cancer Research Institute and Henan Institute of Hematology, the hospital mainly treats patients with various solid tumors and hematological malignancy, receiving critically-ill cancer patients. As one of the first members of China Antimicrobial Resistance Surveillance System (<https://www.carss.cn/>), we standardized participation in national antimicrobial resistance surveillance programs. The CRE strain determination criteria were defined as follows [4]: *Enterobacteriaceae* bacteria that are insensitive to any carbapenem antibiotic; minimum inhibitory concentration (MIC) against meropenem, imipenem, or ertapenem ≥ 2 $\mu\text{g}/\text{mL}$; or *Enterobacteriales* bacteria that are confirmed to produce carbapenemase. Bacterial susceptibility testing and identification using the Phoenix M50 Automated Microbiology System (BD United States/<https://www.bd.com/en-us>) Kirby-Bauer disk diffusion method was used for supplementary antimicrobial susceptibility testing. Antibiotic susceptibility disk purchased from OXIOD Thermo Fisher Scientific (www.thermofisher.com).

Carbapenemase inhibitor buffer reagent (buffer solution CRE) was purchased from Zhuhai DL Biotech ([\[biohema.com/productinfo/2086183.html?templateId=1133605\]\(http://biohema.com/productinfo/2086183.html?templateId=1133605\)\). NG-test CARBA5 Colloidal gold immunoassay agent was purchased from Fosun Diagnostic Technology Co., Ltd.](https://</p>
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(<https://www.fosundiagnosics.com.cn/web/productDetails.do?id=2205031711353990511814&key=wswyq>).

PCR reagents and primers for five common carbapenemase genes (bla_{KPC} , bla_{IMP} , bla_{NDM} , bla_{OXA} , and bla_{VIM}) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (<https://store.sangon.com/product?categoryId=23>). The standard strain ATCC 25922 of *Escherichia coli* was stored in our laboratory; *Klebsiella pneumoniae* producing bla_{KPC} , *Escherichia coli* producing bla_{NDM} , and *Klebsiella pneumoniae* producing both bla_{KPC} and bla_{NDM} were used as experimental quality control strains, which were validated by whole genome sequencing and stored in our laboratory [5].

Identification and drug sensitivity testing of *Enterobacteriaceae*

The identification of CRE strains and drug sensitivity experiments were strictly carried out in accordance with the requirements of the "Common Bacterial Drug Sensitivity Test Report Standardization: Consensus Among Chinese Experts". The *in vitro* antibiotic susceptibility test adopts the micro broth dilution method, and the antibiotics (ciprofloxacin, levofloxacin, cefotaxime, etc.) the Phoenix M50 uncovered were supplemented with antibiotic sensitivity testing using the Kirby-Bauer disk diffusion method. The interpretation of antibiotic susceptibility test results strictly followed the 2022 Clinical and Laboratory Standards Institute, M100, 32nd Edition document (<https://clsi.org/about/news/clsi-publishes-m100-performance-standards-for-antimicrobial-susceptibility-testing-32nd-edition/>).

PCR-based detection of carbapenemase gene

The types of bla_{KPC} , bla_{NDM} , bla_{IMP} , bla_{OXA} , and bla_{VIM} genes were detected by PCR, PCR reaction conditions, and primer sequence [6] (Table 1).

Carbapenemase inhibitor test

The activity of A-class carbapenemase can be inhibited by 3-amino-phenylboronic acid (APB), and the activity of B-class can be inhibited by ethylene diamine tetraacetic acid (EDTA). By referring to the Kirby-Bauer disk diffusion method recommended by CLSI M100, we operated according to the instructions of the buffer solution CRE manual: we prepared 0.5 M suspension of the bacteria and spread it onto MH agar plates, then we applied four sheets of imipenem or meropenem disk evenly on the agar surface within 15 minutes. One plate had none of the reagents added to it and acted as the blank, one plate had 5 μL of APB solution (final concentration 300 $\mu\text{g}/\text{disk}$) dropwise added onto it, another plate had 5 μL of EDTA solution (final concentration 292 $\mu\text{g}/\text{disk}$) dropwise added onto it, and a last plate had 5 μL of APB and 5 μL of EDTA simultaneously added onto it. We incubated them at 35°C for 16 - 18 hours

and measured the zone diameter according to the standard. Result interpretation: If the zone diameter with added APB differed by ≥ 5 mm from the blank, the bacterium produced A-class carbapenemase; if the diameter with EDTA differed by ≥ 5 mm from the blank, the bacterium produced B-class carbapenemase. Only when the zone diameter containing both APB and EDTA differed by ≥ 5 mm from the blank, could it be determined that the strain produced both A and B-class carbapenemase simultaneously. If the difference between the zone diameter with buffer and the blank was less than 5 mm, the bacterium did not produce class A nor B carbapenemase.

NG-test CARBA5

We operated according to the instructions of the NG-test CARBA5 kit. In order to ensure the test results were not affected or interfered with, the operators were blinded to the PCR results during the carbapenemase inhibitor or NG-test CARBA 5 interpretation.

Statistical analysis

Bacterial distribution and antibiotic sensitivity results were statistically analyzed using WHONET 5.6 (www.whonet.org.cn). By using PCR results as a reference, we calculated the sensitivity and specificity of carbapenemase inhibitor assay and NG-test CARBA5.

Sensitivity = true positive/(true positive + false negative) $\times 100\%$,

specificity = true negative/(true negative + false positive) $\times 100\%$.

RESULTS

Detection and antibiotic sensitivity of CRE strains

A total of 124 unduplicated CRE strains were detected in hospitalized patients at Henan Cancer Hospital in 2022, including 62 *Klebsiella pneumoniae* strains (50%), 30 *Escherichia coli* strains (24.1%), 12 *Escherichia cloacae* strains (9.7%), 8 *Klebsiella oxytoca* strains (6.5%), 8 *Citrobacter* strains (6.5%), and 4 *Serratia marcescens* strains (3.2%). CRE strains have a high resistance rate to common antibiotics. CRE strains have a resistance rate of 100% to imipenem and 94% to meropenem and relatively lower resistance rates to tigecycline, polymyxin, amikacin, and cefotaxime/avibactam. Resistance rates to azurone, ciprofloxacin, and levofloxacin lay around 60% (Supplementary Material 1).

PCR carbapenemase detection results

The test results for 124 positive strains are as follows: 69 bla_{KPC}-positive, 48 bla_{NDM}-positive, 1 bla_{IMP}-positive, and 4 bla_{KPC} + bla_{NDM} double-positive. No bla_{OXA}- or bla_{VIM}-positive strains were detected. Two strains out of the 124 strains showed negative results for five common carbapenemases. The electrophoresis results of some PCR products are shown in Figure 1.

NG-test CARBA5 results

Among 124 CRE strains, 117 strains were positive, including 68 bla_{KPC} strains, 43 bla_{NDM} strains, and 1 bla_{IMP} strain; 4 strains simultaneously producing bla_{KPC} and bla_{NDM}, 1 strain simultaneously producing bla_{OXA} and bla_{NDM}, and no bla_{VIM} strain were detected (Supplementary Material 2).

Carbapenemase inhibitor results

Among 124 strains, 122 strains were positive, including 70 strains producing A-class carbapenemase, 49 strains producing B-class carbapenemase, 3 strains simultaneously producing class A and B carbapenemase (Supplementary Material 3).

Comparison of carbapenemase inhibitor and NG-test CARBA5

Based on the PCR results, the carbapenemase inhibition test had a sensitivity of 100.00% and a specificity of 98.36%. The positive rate of NG-test CARBA5 for five common carbapenemases was 95.90%, with a specificity of 99.18%. Both methods showed good sensitivity and specificity (Table 2).

DISCUSSION

The national bacterial resistance monitoring results released by CHINET in 2025 show that the resistance rate of *Klebsiella pneumoniae* to carbapenem antibiotics has significantly increased from around 3% in 2005 to around 25% in 2023; a 7-fold increase. The accurate identification of CRE strains and carbapenemase types is of great significance for anti-infection and prevention of CRE transmission. A total of 124 unduplicated CRE strains were detected in our hospital in 2022, including *Klebsiella pneumoniae*, *Escherichia coli*, *Escherichia cloacae*, *Klebsiella oxytoca*, *Citrobacter*, and *Serratia marcescens*. There was not much difference in data compared to other similar hospitals [7]. The PCR method for detecting the carbapenemase was the golden standard for CRE strains, but it has not been routinely carried out in hospitals [8].

We noticed that carbapenemase inhibitor assays can clearly distinguish between A-class and B-class carbapenemase and can detect non-carbapenemase producing strains. For detecting isolates co-producing *Klebsiella pneumoniae* carbapenemase (KPC) and metallo- β -lactamases (MBLs), the carbapenemase inhibitor assays had higher sensitivity [9]. However, this method has a common problem with phenotype-based detection of carbapenemases, which is that the accuracy of detection is directly related to the resistance level of CRE strains to carbapenem antibiotics; low level resistance may lead to false negative results. We have strong reason to suggest that molecular biology methods continue to be used to detect CRE strains. In addition, carbapenemase inhibitor assays were ineffective for CRE strains that only produce bla_{OXA-48}.

Table 1. PCR primer sequences of five common carbapenemases.

Carbapenemases	Forward primer 5'-3'	Reverse primer 5'-3'
bla_{KPC}	CGCGGAACCATTCGCTAA	CGGTATCCATCGCGTACACA
bla_{NDM}	CGTGCTGGTGGTCGATACC	CCTGCTTGATCCAGTTGAGGAT
bla_{IMP}	GGAATAGAGTGGCTTAATTCTC	GGTTTAATAAAAACAACCACC
bla_{OXA}	GATGGACAGACGCGGATA	ACTGAATATTTTCATCGCGGTGAT
bla_{VIM}	CGCTTCGGTCCAGTAGAGCTCT	CCACCGTATAGCACGTTTCGCTG

Table 2. Comparison of carbapenemase results of carbapenemase inhibitor test and NG-test CARBA5.

CRE strains	n	PCR		NG-test CARBA5			Carbapenemase inhibitor test			
		Carba-penemase	n	n	Sensitivity	Specificity	Carba-penemas	n	Sensitivity	Specificity
<i>Klebsiella pneumoniae</i>	62	KPC	40	39	97.50%	100.00%	A-class	40	100.00%	100.00%
		NDM	19	18	94.74%	100.00%	B-class	20	100.00%	94.74%
		KPC + NDM	2	2	100.00%	100.00%	Both A and B	1	50.00%	100.00%
<i>Escherichia coli</i>	30	KPC	4	4	100.00%	100.00%	A-class	4	100.00%	100.00%
		NDM	24	22	91.67%	100.00%	B-class	23	95.83%	100.00%
		KPC + NDM	1	1	100.00%	100.00%	Both A and B	1	100.00%	100.00%
		OXA + NDM	1	1	100.00%	100.00%	B-class	1	100.00%	100.00%
<i>Escherichia cloacae</i>	12	KPC	8	9	100.00%	87.50%	A-class	8	100.00%	100.00%
		NDM	3	2	66.67%	100.00%	B-class	4	100.00%	100.00%
		IMP	1	1	100.00%	100.00%				
<i>Klebsiella oxytoca</i>	8	KPC	7	7	100.00%	100.00%	A-class	7	100.00%	100.00%
		NDM	1	1	100.00%	100.00%	B-class	1	100.00%	100.00%
<i>Citrobacter</i>	8	KPC	6	6	100.00%	100.00%	A-class	7	100.00%	83.33%
		KPC + NDM	1	1	100.00%	100.00%	Both A and B	1	100.00%	100.00%
<i>Serratia marcescens</i>	4	KPC	4	3	75.00%	100.00%	A-class	4	100.00%	100.00%
Total	124		122	117	95.90%	99.18%		122	100.00%	98.36%

The carbapenemase inhibition test has a sensitivity of 100.00% and a specificity of 98.36%. The positive rate of NG-test CARBA5 was 95.90%, with a specificity of 99.18%. Both methods show good sensitivity and specificity.

A few studies, including ours, confirmed that NG-test CARBA 5 exhibits more than 95% sensitivity and nearly 100% specificity, demonstrating superior performance in identifying five common carbapenemase types [9]. The limitation of NG-test CARBA 5 is that it has no ability to detect carbapenemases other than the five common types and that it is insensitive to rare subtypes such as bla_{OXA-204} and bla_{OXA-370}. Fortunately, the clinical detection rates of bla_{OXA-204} and bla_{OXA-370} were very low. Limited by the small sample size and single-center

research, we could not get enough strain of bla_{OXA} subtype. Whether the carbapenemase inhibitor assay was sensitive to bla_{OXA-204} and bla_{OXA-370} had not been confirmed. This may be our research direction in the future. The detection of bla_{OXA-48} poses many complicated situations: there are many bla_{OXA} subtypes, and the distribution of bla_{OXA} often exhibits regional characteristics globally [10]. Also, cross-reactions often arise and are difficult to avoid. Occasionally, there is a situation as follows: microbial antibiotic sensitivity indicates CRE,

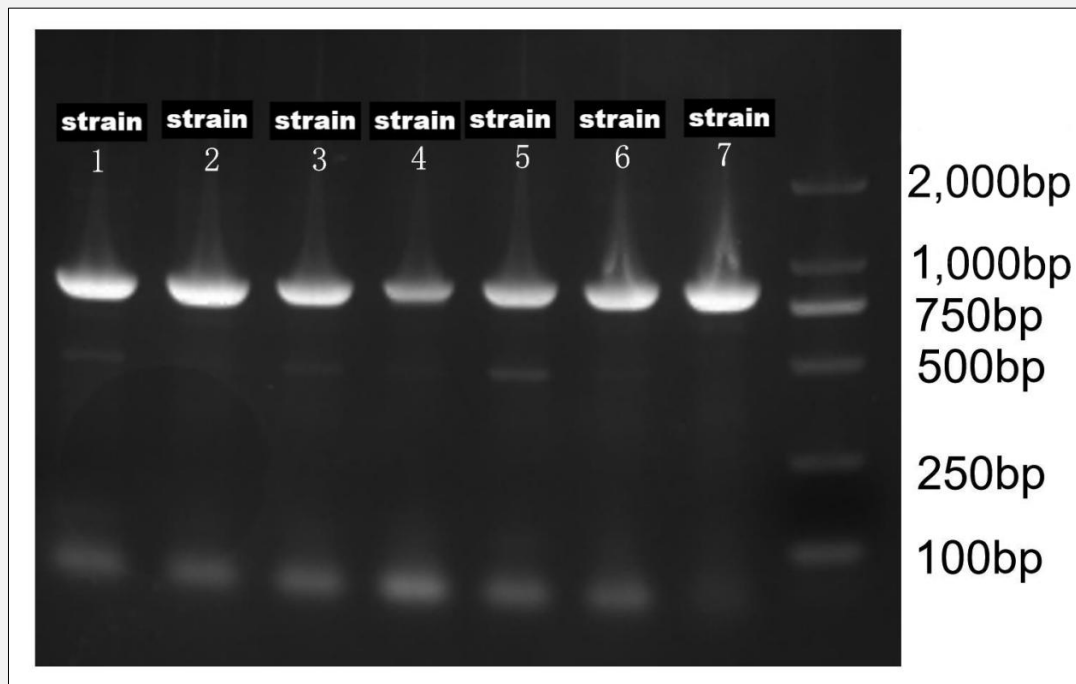


Figure 1. The electrophoresis results of some PCR productions.

The length of five common carbapenemase genes, *bla_{KPC}* (870 - 900 bp), *bla_{IMP}* (750 - 800 bp), *bla_{NDM}* (800 - 900 bp), *bla_{OXA}* (740 - 800 bp), and *bla_{VIM}* (780 - 810 bp), is between 750 - 1,000 bp.

carbapenemase inhibitor detection was negative, NG-test CARBA5 method positive, which indicated that the strain may produce *bla_{OXA-48}*, since NG-test CARBA5 method has excellent detection ability for *bla_{OXA-48}* [11]. In the daily clinical detection of carbapenemase, we found that there are very few cases of low concentration resistance to carbapenem antibiotics leading to negative phenotype detection of carbapenemase inhibitor detection.

Cross-reaction and the emergence of carbapenemase subtypes make detection more and more important and complex. Under the selective pressure of antibiotics, the emergence of new KPC subtypes such as *bla_{KPC-33}*, *bla_{KPC-76}*, and *bla_{KPC-51}* can cause false negatives in laboratory routine carbapenemase detection [12]. It must be acknowledged that both carbapenemase inhibitor test and NG-test CARBA5 are insufficient for the detection and resolution of carbapenemase subtypes. Molecular biology such as PCR or whole-genome sequencing has better advantages in subtype detection.

There are several inconsistent results regarding carbapenemase detection: If carbapenemase inhibitor test is positive but NG-test CARBA5 is negative, it indicates that the bacterium produces rare carbapenemase, other than the five common carbapenemases. If the carba-

penemase inhibitor test and NG-test CARBA5 are both negative, it indicates that the strain is a non-carbapenemase-producing strain. The resistance mechanism may be the production of extended spectrum β -lactase or AmpC combined with downregulation of outer membrane protein expression.

CONCLUSION

Our research has shown that the carbapenemase inhibition test has a sensitivity of 100.00% and a specificity of 98.36. The positive rate of NG-test CARBA5 for five common carbapenemases was 95.90%, with a specificity of 99.18%. In conclusion, the NG-test CARBA5 has been proven to be a reliable method in detecting carbapenemase, and the carbapenemase inhibition test offers a practical and cost-effective approach for their detection, suitable for widespread use in laboratories.

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Ethical Approval Statement:

The study was approved by the Ethics Committee of the Affiliated Cancer Hospital of Zhengzhou University. The ethical approval number is LHGJ20230101.

Additional material can be found online at:

<http://supplementary.clin-lab-publications.com/250632/>

Declaration of Interest:

The authors declare that they have no conflicts of interest. All authors participated in the discussion, provided conceptual input, and have read and approved the final manuscript.

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