

Application of Modified Carbapenem Inactivation Method and Its Derivative Tests for the Detection of Carbapenemase-Producing *Aeromonas*

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Purpose: Infection and transmission of carbapenem-resistant *Aeromonas* is a serious threat to public health. Rapid and accurate detection carbapenem-resistant of these organisms is essential for reasonable treatment and infection control. This study aimed to find a simple and effective method to detect carbapenem-resistant phenotype in *Aeromonas*.

Methods: A total of 131 clinical preserved *Aeromonas* strains were used in this study. The carbapenemase genes were detected by PCR. Modified carbapenem inactivation method (mCIM) in conjunction with EDTA-modified carbapenem inactivation method (eCIM) and simplified carbapenem inactivation method (sCIM) were performed to detect carbapenemases. We also designed a simple method, carbapenem inactivation method using supernatant (CIM-s), to detect the carbapenemase activity in the medium.

Results: Of the 131 *Aeromonas* strains, 79 contained carbapenemase genes, including 68 *bla*_{CphA}, 6 *bla*_{KPC-2}, 2 *bla*_{NDM-1} and 3 *bla*_{KPC-2+CphA}. However, routine antibiotic susceptibility testing could not completely identify carbapenemase-producing *Aeromonas*. In phenotypic assays, the sensitivity and specificity of mCIM were 100%. The combined mCIM and eCIM could distinguish serine carbapenemase and metallo-β-carbapenemases except co-producing organisms. The sensitivity and specificity of sCIM were 92.4% and 100%, respectively, which could not detect CphA totally. CIM-s results indicate that these carbapenemases could secrete into the medium to perform their hydrolytic activities and had a sensitivity and specificity of 97.5% and 100%, respectively.

Conclusion: The combination of mCIM and eCIM can effectively detect and distinguish different types of carbapenemase in *Aeromonas*, and could be used as an important supplement approach to the antibiotic susceptibility testing.

Keywords: *Aeromonas*, modified carbapenem inactivation method, carbapenemase, multidrug resistant, phenotypic detection

Introduction

Bacterial resistance has become an urgent global concern. The increase in multidrug-resistant and even pan drug-resistant bacteria has brought serious challenges to clinical diagnosis and treatment.¹ Gram-negative bacteria are important opportunistic pathogens of community-acquired infection and hospital-acquired infection. With the emergence of carbapenem-resistant Gram-negative bacteria, like non-fermenting Gram-negative bacteria and *Enterobacteriales*, the clinical treatment options are very limited, which in turn prolong the length of stays in hospital, increase the cost of care and enhance the risk of mortality.²⁻⁴ Currently, it is widely accepted that the significant

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mechanism of carbapenem resistance in these organisms is producing carbapenemases. Carbapenemases are mainly categorized into Ambler class A, B, and D depending on the Ambler classification scheme. Class A and D carbapenemases are serine carbapenemases with serine hydrolysis mechanism. In addition, class B carbapenemases are metallo- β -lactamases (MBLs) requiring zinc ions catalysis to generate hydrolysis activity.^{4,5} Carbapenemase genes are frequently located on plasmids and mobile genetic elements, facilitating transfer between different strains, which make nosocomial infection control facing severe challenges.^{6,7}

The genus *Aeromonas*, widely distributed in the natural environment, is a Gram-negative, oxidase-positive, facultative-anaerobic bacillus. There are 36 species in the genus *Aeromonas*,⁸ of which *A. hydrophila*, *A. caviae* and *A. veronii* bv. *sobria* are the major pathogens of infection.⁹ In addition to diarrhea, *Aeromonas* can cause a variety of extra-intestinal infections, such as skin and soft tissue infection, wound infection, intra-abdominal infection, pneumonia and bacteremia, indicating that *Aeromonas* isolates are significant human opportunistic pathogens.^{10–14} Therefore, infection and transmission of carbapenem-resistant *Aeromonas* pose a potential threat to public health. The main mechanism of *Aeromonas* resistance to carbapenems is to carry CphA MBL that is located in the chromosome and only has activity against carbapenems.^{15,16} Besides CphA, *Aeromonas* strains carrying other carbapenemases are also emerging. In 2007, *Aeromonas caviae* containing IMP-19 MBL was isolated in France.¹⁷ In 2008, the first multi-resistant *Aeromonas hydrophila* producing VIM-4 MBL was identified in Hungary.¹⁸ Additionally, *Aeromonas*-producing KPC-2 carbapenemase also successively emerged in Brazil, the United States and China.^{19–21} In recent years, *Aeromonas* harboring *bla*_{OXA-181}, *bla*_{GES-24} and even *bla*_{GES/OXA-48/NDM-1} multiple carbapenemase-encoding genes have been detected.^{22–24}

Molecular methods to detect carbapenemase-encoding genes are regarded as the gold standard for identification of carbapenemase-producing organisms. However, molecular methods require professional technicians, special detection equipment, expensive costs, time-consuming and multitudinous potential target genes, which limit their routine application.^{3,6,25} Various phenotypic assays based on carbapenemase characteristics have been developed, including modified Hodge test, Carba NP test, modified carbapenem inactivation method (mCIM), matrix-assisted laser

desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), and other derivative experiments, which can be carried out in microbiological laboratory.^{4–6} For *bla*_{CphA}-positive *Aeromonas*, the antimicrobial susceptibility testing by disk diffusion or MIC failed to fully display carbapenem-resistant phenotype. On the contrary, some phenotypic assays yield consistent results with genotypes.^{15,16} Here, it should be noted that the mCIM in conjunction with EDTA-modified carbapenem inactivation method (eCIM), recommended by CLSI, reveals perfect sensitivity and specificity in carbapenemase-producing *Enterobacterales*.^{5,26,27} However, whether mCIM and eCIM are suitable for the detection of carbapenemase in *Aeromonas* remains to be established.

In the present study, we systematically analyzed the antimicrobial resistance patterns of *Aeromonas* and compared the consistency of different methods for the antimicrobial susceptibility of carbapenems. Then, we detected the production of carbapenemase in *Aeromonas* by mCIM in conjunction with eCIM, simplified carbapenem inactivation method (sCIM),²⁸ and compared them with polymerase chain reaction (PCR) to investigate the potential resistance mechanisms to carbapenems. To the best of our knowledge, this is the first confirmation that mCIM and eCIM are accurate and effective for the detection of multiple carbapenemases in *Aeromonas*. Moreover, we designed a simple method, carbapenem inactivation method using supernatant (CIM-s), to detect the activity of carbapenemase secreted in the medium.

Materials and Methods

Bacterial Isolates and Antibiotic Susceptibility Testing

A total of 131 clinical preserved *Aeromonas* strains from The Second Affiliated Hospital of Chongqing Medical University were used in this study. These isolates were mainly from the liver abscess drainage (36/131), followed by wound secretions (19/131), blood (18/131), pus (14/131), stool (13/131), bile (11/131), urine (8/131), sputum (7/131), and ascitic fluid (5/131). All isolates were stored in skim milk supplemented with 10% glycerine at -80°C . And these strains were subcultured twice at 35°C for 18–24 h using blood agar plates before testing. The organisms were identified to the species level by Vitek-2 Compact system (bioMérieux, Hazelwood, MO, USA) and were confirmed by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions. The antibiotic

susceptibility testing was performed by using Vitek-2 AST-GN67 and AST-XN04 cards (bioMérieux, Hazelwood, MO, USA). As the CLSI guideline M45 recommended, the susceptibilities of imipenem and meropenem were confirmed by the disk diffusion method on Mueller–Hinton agar (MHA) and broth microdilution method using cation-adjusted Mueller–Hinton broth (CAMHB).²⁹ The results were interpreted according to the CLSI guideline M45.²⁹ This study does not include any patient information.

Molecular Identification of Carbapenemase-Encoding Genes by PCR

For analysis of carbapenemase-encoding genes, bacterial genomic DNA was extracted using a Spin Column Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). Then, PCR was performed using gene-specific primers and PCR kit (Takara Bio Inc. Otsu, Japan) in BIO-RAD MyCycler PCR system (Bio-Rad Laboratories, Inc., California, USA). The PCR thermal cycling procedures were as follows: 30 seconds at 94°C for enzyme activation,

35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, finishing with a 10-minute step at 72°C for final extension. The sequences and product lengths of primers are summarized in Table 1. The PCR amplified products were analyzed by electrophoresis on 1.5% agarose gels and visualized in a BIO-RAD Gel Doc XR+ gel imaging system (Bio-Rad Laboratories, Inc., California, USA). All positive products of the carbapenemase-encoding genes were verified by commercial direct sequencing (Sangon Biotech, Shanghai, China).

mCIM in Conjunction with eCIM for Carbapenemase Production in *Aeromonas*

The mCIM and eCIM for *Aeromonas* were performed according to the method recommended by CLSI for *Enterobacteriales*.³⁰ Two 1-μL loopfuls of bacteria from an overnight blood agar plate were emulsified in 2 mL trypticase soy broth (TSB) or 2 mL TSB added 20 μL of the 0.5 M EDTA, respectively. Subsequently, a 10-μg meropenem disk (Oxoid Ltd, Basingstoke, UK) was

Table 1 Primer Sequences for Carbapenemase-Encoding Genes

Gene	Primer	Nucleotide Sequence (5'–3')	Size (bp)
CphA	F R	GCTTAGAGCTCCTAAGGAGCAAGATGAAAGGTTGG GCATAGGTACCTTATGACTGGGGTGCGGCCCTTG	720
KPC-2	F R	CATTCAAGGGCTTTCTTGCTGC ACGACGGCATAGTCATTTGC	538
NDM-1	F R	CAGCACACTTCCTATCTC CCGCAACCATCCCCCTCTT	292
VIM	F R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390
SME	F R	AACGGCTTCATTTTGTGTTAG GCTTCCGCAATAGTTTATCA	820
IMP	F R	CATGGTTTGGTTGTTCTTGT ATAATTTAGCGGACTTTGGC	488
GES	F R	GCTTCATTCACGCACTATT CGATGCTAGAAACCGCTC	323
IMI	F R	TGCGGTCGATTGGAGATAAA CGATTCTTGAAGCTTCTGCG	399
GIM	F R	CGAATGGGTTGGTAGTTCTGGATAATAATC ATGTGTATGTAGGAATTGACTTTGAATTTAGC	198
SIM	F R	TACAAGGGATTGCGCATCG TAATGGCCTGTTCCCATGTG	571
OXA-48-like	F R	TTGGTGGCATCGATTATCGG GAGCACTTCTTTGTGATGGC	438

immersed in each suspension and incubated at 35°C in ambient air for 4 h. A MHA plate was inoculated with a 0.5 McFarland suspension of *E. coli* ATCC 25922 as for the routine disk diffusion procedure. The meropenem disks were separately removed from the TSB or TSB-EDTA suspension and placed on the same MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC 25922 indicator strain. All plates were incubated at 35°C in ambient air for 18–24 h. Following incubation, the zones of inhibition were measured as for the routine disk diffusion method. The results were interpreted according to the CLSI guideline M100 for mCIM and eCIM in *Enterobacterales*.³⁰ The mCIM and eCIM using 10-μL loopful of *Aeromonas* were also carried out. The test was performed in triplicate, and the results were interpreted by three independent technicians.

sCIM for Carbapenemase Production in *Aeromonas*

According to the method described by Jing et al,²⁸ we performed sCIM for carbapenemase production in *Aeromonas*. A MHA plate was inoculated with a 0.5 McFarland suspension of *E. coli* ATCC 25922 as for the routine disk diffusion procedure. One to three colonies of test organisms from an overnight blood agar plate were smeared onto one side of 10-μg imipenem disk (Oxoid Ltd, Basingstoke, UK). Then, the side of imipenem disk containing test bacteria was placed on the MHA plate previously inoculated with the imipenem-susceptible *E. coli* ATCC 25922 indicator strain. An imipenem disk without any bacteria was placed on the same MHA plate as the control. After the plates were incubated at 35°C in ambient air for 16–18 h, the zones of inhibition were measured as for the routine disk diffusion method. A zone diameter of 6–20 mm or presence of pinpoint colonies within a ≤22 mm zone diameter was considered to be carbapenemase positive. A zone diameter of ≥26 mm was considered to be carbapenemase negative. A zone diameter of 23–25 mm was considered to be carbapenemase indeterminate.²⁸ All strains were tested in triplicates, and the results were interpreted by three independent technicians.

CIM-s for the Detection of Carbapenemase Activity in the Medium

A 10-μL loopful of bacteria from an overnight blood agar plate were emulsified in 10 mL TSB and incubated at 35°C in ambient air for 18–24 h. The suspension was

centrifuged at 8000 rpm for 10 min to collect the supernatant. A 10-μg meropenem disk was immersed in each 2 mL centrifuged supernatant or 2 mL supernatant added 20 μL of the 0.5 M EDTA, respectively, instead of incubating the disk in the organism suspension as in the mCIM and eCIM. After incubation at 35°C in ambient air for 1 h, 2 h and 4 h, respectively, the meropenem disks were separately removed from the supernatant or supernatant-EDTA and placed on the MHA plate previously inoculated with a 0.5 McFarland suspension of *E. coli* ATCC 25922. Then, the plates were incubated at 35°C in ambient air for 18–24 h. The zone diameters were measured as for the routine disk diffusion method. A meropenem disk, immersed in 2 mL TSB, was placed on the MHA plate as the control. And, 100 μL of supernatant was inoculated onto the blood agar plate to check that the culture supernatants were bacteria free. The results were interpreted according to the CLSI M100 for mCIM and eCIM in *Enterobacterales*.³⁰ The test was performed in triplicate, and three independent technicians interpreted the results.

Statistical Analysis

Statistical analyses were performed with the SPSS software version 22.0 (SPSS, Inc., Chicago, IL, USA). Descriptive statistics were presented as number and percentage. McNemar's test was used to compare the difference of resistance rates of imipenem and meropenem detected by different methods. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy value were calculated using genes' results as the gold standard. The indeterminate results were considered false negative in this study.

Results

Antibiotic Susceptibility of Carbapenem in *Aeromonas*

All 131 isolates were identified as *A. hydrophila*, *A. caviae* and *A. sobria* by Vitek-2 Compact system, and further confirmed as *A. hydrophila*, *A. caviae* and *A. veronii* by MALDI-TOF MS. However, the species results of the two methods were not completely consistent. The isolates demonstrated high resistance to imipenem (54.96%), meropenem (46.56%) and doripenem (43.51%), but were less so for ertapenem (17.56%). We also determined the susceptibilities of two main carbapenems, imipenem and meropenem, by the disk diffusion

method and broth microdilution method. The resistance rates to imipenem and meropenem were 34.35% and 8.40% by the disk diffusion, and were 30.53% and 9.92% by the broth microdilution, respectively. The intermediate rates were dramatically increased. The antibiotic susceptibility results of imipenem and meropenem among the three methods were significant different (Tables 2 and 3, $\chi^2=30.719$, $p<0.001$ for imipenem and $\chi^2=72.971$, $p<0.001$ for meropenem).

Aeromonas Harboring Diverse Carbapenemase-Encoding Genes

We detected the main carbapenemase-encoding genes, including class A carbapenemases (*bla_{KPC-2}*/*bla_{IMP}*/*bla_{GES}*), class B carbapenemases (*bla_{NDM-1}*/*bla_{IMP}*/*bla_{VIM}*/*bla_{CphA}*) and class D carbapenemases (*bla_{OXA-48-like}*), by PCR. Of the 131 isolates, 79 isolates (60.3%) contained carbapenemase genes, and 52 isolates (39.7%) were carbapenemase negative without any of the tested carbapenemase

genes. Of the 79 carbapenemase-positive isolates, 68 harbored the carbapenemase genes *bla_{CphA}*, 6 contained *bla_{KPC-2}*, 2 harbored *bla_{NDM-1}* and 3 were positive for both *bla_{KPC-2}* and *bla_{CphA}* simultaneously. The PCR-positive products of the carbapenemase-encoding genes were verified by sequencing.

mCIM in Conjunction with eCIM Can Accurately Detect Carbapenemase in Aeromonas

Of the 131 isolates, 79 isolates were carbapenemase positive and 52 isolates were carbapenemase negative by mCIM that were consistent with the PCR-based gene testing results. The inhibition zone diameters of 52 non-carbapenemase producers were 21–23 mm. In 79 PCR-positive isolates, 68 *Aeromonas* with *bla_{CphA}* and 2 *Aeromonas* with *bla_{NDM-1}* exhibited mCIM and eCIM positive. Six *Aeromonas* contained *bla_{KPC-2}* and three *Aeromonas* harbored both *bla_{KPC-2}* and *bla_{CphA}* displayed mCIM positive and eCIM negative (Figure 1A–C and Table 4). Both sensitivity and specificity of mCIM were 100% (Table 5). The test results using 1-μL and 10-μL loopfuls of bacteria were identical (data not shown).

Table 2 Distribution of Imipenem Results Detected by Different Methods (n)

PCR (n)	Vitek-2 Compact (mg/L)			Broth Microdilution (mg/L)			Disk Diffusion (mm)		
	≥4	2	≤1	≥4	2	≤1	≤19	20–22	≥23
Positive (79)	72	3	4	40	24	15	45	26	8
CphA (68)	61	3	4	29	24	15	34	26	8
KPC-2 (6)	6	0	0	6	0	0	6	0	0
NDM-1 (2)	2	0	0	2	0	0	2	0	0
KPC-2+CphA (3)	3	0	0	3	0	0	3	0	0
Negative (52)	0	0	52	0	0	52	0	0	52

Table 3 Distribution of Meropenem Results Detected by Different Methods (n)

PCR (n)	Vitek-2 Compact (mg/L)			Broth Microdilution (mg/L)			Disk Diffusion (mm)		
	≥4	2	≤1	≥4	2	≤1	≤19	20–22	≥23
Positive (79)	68	2	9	13	7	59	11	7	61
CphA (68)	57	2	9	2	7	59	0	7	61
KPC-2 (6)	6	0	0	6	0	0	6	0	0
NDM-1 (2)	2	0	0	2	0	0	2	0	0
KPC-2+CphA (3)	3	0	0	3	0	0	3	0	0
Negative (52)	0	0	52	0	0	52	0	0	52

The Sensitivity and Specificity of sCIM

The zone diameters of 52 carbapenemase gene-negative isolates were all ≥26 mm. In 79 carbapenemase gene-positive isolates, 73 isolates of zone diameters were 6–20 mm or presence of pinpoint colonies within a ≤22 mm zone diameter that was considered to be carbapenemase positive by sCIM. Among the 73 isolates that were positive by sCIM, the zone diameters of *Aeromonas* expressing *bla_{KPC-2}*, *bla_{NDM-1}* and *bla_{KPC-2}+CphA* were all 6mm. However, the zone diameters of *Aeromonas*, harboring *bla_{CphA}* alone, ranged from 6 to 20 mm. The other six gene-positive isolates displayed the zone diameters of 23–25 mm that were considered to be carbapenemase indeterminate (Figure 2 and Table 4). The carbapenemase indeterminate isolates were all *bla_{CphA}* positive. The sensitivity and specificity were 92.4% and 100%, respectively (Table 5).

The Results of CIM-s in Aeromonas

According to the results of CIM-s, we speculated that KPC-2, NDM-1 and CphA were secreted carbapenemases. The culture supernatants of isolates that were positive for carbapenemase genes were screened for secreted

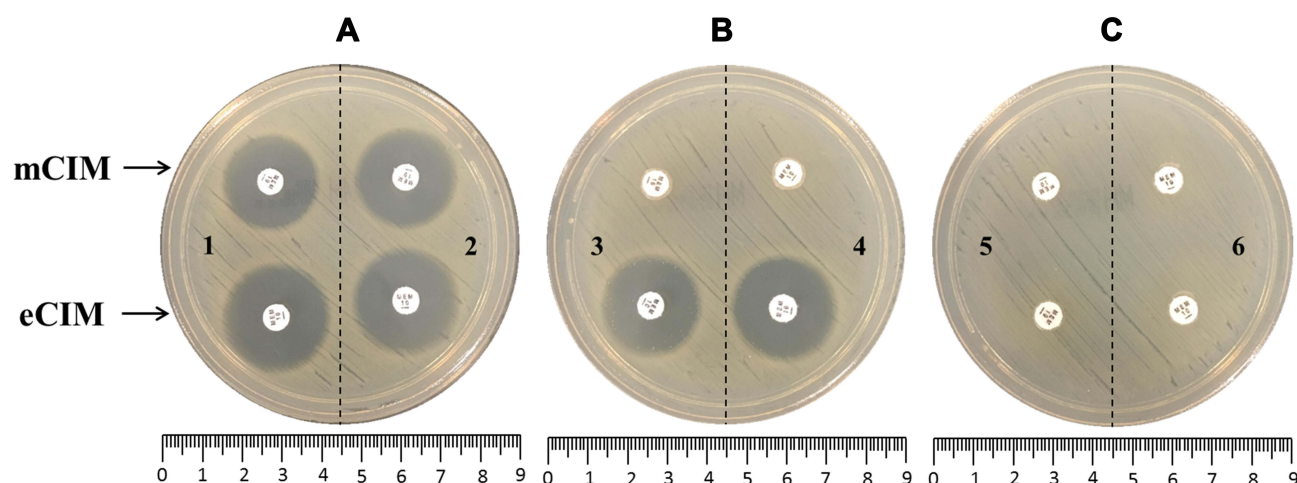


Figure 1 The representative photograph of mCIM and eCIM results in *Aeromonas*. **(A)** Negative results. 1, blank control, without bacteria; 2, *Aeromonas* without carbapenemase production. **(B)** mCIM positive and eCIM positive. 3, *Aeromonas*-producing CphA; 4, *Aeromonas*-producing NDM-1. **(C)** mCIM positive and eCIM negative. 5, *Aeromonas*-producing KPC-2; 6, *Aeromonas*-producing both KPC-2 and CphA.

Abbreviations: mCIM, modified carbapenem inactivation method; eCIM, EDTA-modified carbapenem inactivation method.

carbapenemase activity. With the prolongation of incubation time, the zone diameters were decreased. After incubation for 1 h, the zone diameters of 36 isolates were in the positive range. After incubation for 2 h, the positive results increased to 58 strains. At the end of 4 h incubation, 77 isolates displayed carbapenemase activity,

yet there were still two isolates with *bla*_{CphA} that showed indeterminate results. Adding 0.5M EDTA, the zone diameters of 66 isolates with *bla*_{CphA} and 2 isolates with *bla*_{NDM-1} were expanded ≥ 5 mm. Six isolates with *bla*_{KPC-2} and 3 isolates with *bla*_{KPC-2+CphA} had no significant change with the zone diameters expanding < 4 mm. In this

Table 4 Comparison of mCIM, sCIM and CIM-s in the Detection of Carbapenemase

PCR (n)	Zone (mm)			Positive (n, %)			Indeterminate (n, %)		
	mCIM	sCIM	CIM-s*	mCIM	sCIM	CIM-s*	mCIM	sCIM	CIM-s*
Positive (79)	6–10	6–25	6–20	79(100)	73(92.4)	77(97.5)	0(0)	6(7.6)	2(2.5)
CphA (68)	6–10	6–25	6–20	68(100)	62(91.2)	66(97.1)	0(0)	6(8.2)	2(2.9)
KPC-2 (6)	6	6	6	6(100)	6(100)	6(100)	0(0)	0(0)	0(0)
NDM-1 (2)	6	6–10	6	2(100)	2(100)	2(100)	0(0)	0(0)	0(0)
KPC-2+CphA (3)	6	6	6	3(100)	3(100)	3(100)	0(0)	0(0)	0(0)
Negative (52)	21–23	26–30	21–23	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

Notes: *The results were determined after incubation at 35°C in ambient air for 4h.

Abbreviations: mCIM, modified carbapenem inactivation method; sCIM, simplified carbapenem inactivation method; CIM-s, carbapenem inactivation method using supernatant.

Table 5 Sensitivity and Specificity of Phenotypic Tests and IMP (95% CI)

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
mCIM	100 (95.4–100)	100 (93.2–100)	100 (95.4–100)	100 (93.2–100)	100 (97.2–100)
sCIM	92.4 (84.2–97.2)	100 (93.2–100)	100 (95.1–100)	89.7 (78.8–96.1)	95.4 (90.3–98.3)
CIM-s*	97.5 (91.2–99.7)	100 (93.2–100)	100 (95.3–100)	96.3 (87.3–99.6)	98.5 (94.6–99.8)
IMP**	91.1 (82.6–96.4)	100 (93.2–100)	100 (95.0–100)	88.1 (77.1–95.1)	94.7 (89.3–97.8)

Notes: *The results were determined after incubation at 35°C in ambient air for 4 h. **Resistance to imipenem tested by Vitek-2 compact system.

Abbreviations: mCIM, modified carbapenem inactivation method; sCIM, simplified carbapenem inactivation method; CIM-s, carbapenem inactivation method using supernatant; IMP, imipenem; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

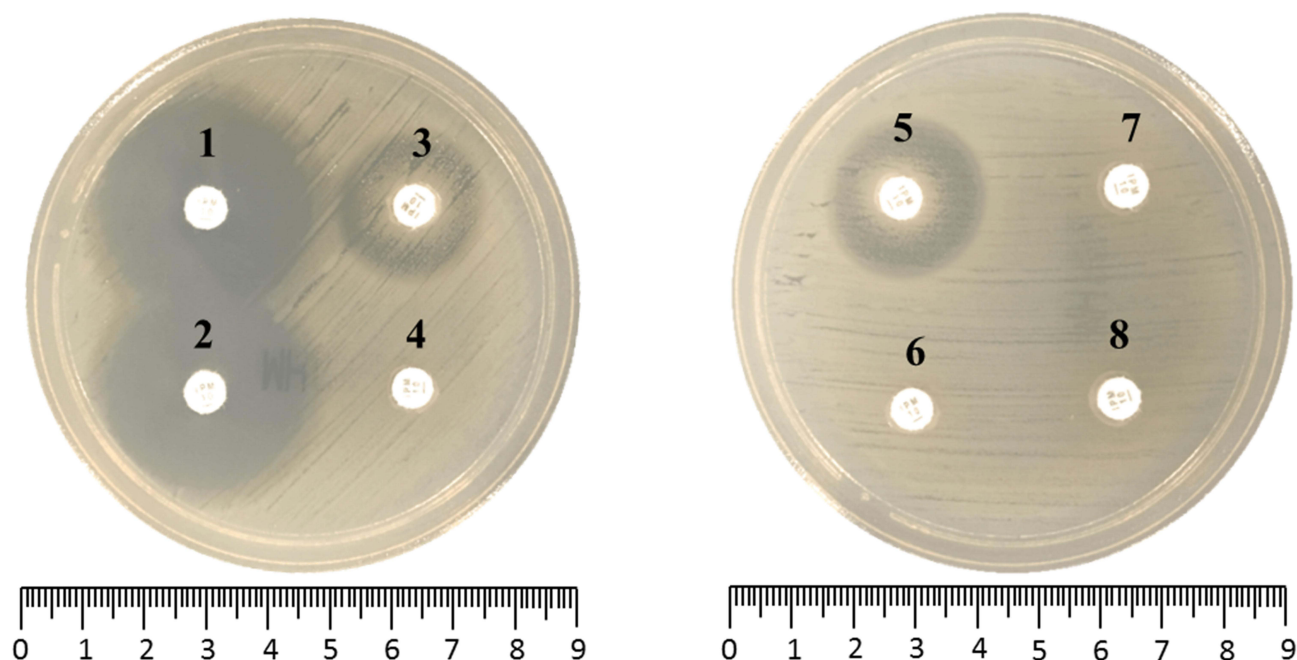


Figure 2 The representative photograph of sCIM results in *Aeromonas*. 1, blank control. 2, negative result, non-carbapenemase-producing *Aeromonas*. 3–4, positive results, CphA-producing *Aeromonas*. 5, indeterminate result, CphA-producing *Aeromonas*. 6–8, positive results, *Aeromonas*-producing KPC-2 (6), NDM-1 (7) or KPC-2+CphA (8). **Abbreviation:** sCIM, simplified carbapenem inactivation method.

process, the zone diameters of 52 isolates without carbapenemase genes were larger than 21 mm, which was interpreted as carbapenemase negative (Figure 3A–F and Table 4). The CIM-s results after 4 h incubation were more consistent with carbapenemase gene testing results. The sensitivity reached 97.5%, and the specificity was 100% (Table 5).

Discussion

The taxonomy of the genus *Aeromonas* is quite complicated. It is difficult to accurately identify *Aeromonas* to species level by traditional biochemical tests and automated systems,^{31,32} which were confirmed by evaluating the accuracy of six commercial systems for identifying clinical *Aeromonas* isolates.³³ MALDI-TOF MS is a rapid and effective method for identification of *Aeromonas* genus. However, the species level results are inconsistent with the housekeeping gene sequencing.^{34,35} In this study, we utilized Vitek-2 Compact system and MALDI-TOF MS to identify 131 clinical isolates as *Aeromonas*, although there were some differences at the species level. In subsequent studies, we did not further distinguish the species, referring to all isolates as *Aeromonas*.

The carbapenemase gene detection results demonstrated that *bla*_{CphA}, accounting for 60%, was the major carbapenemase gene in *Aeromonas*. In other studies, the

prevalence of *bla*_{CphA} was even higher, reaching 69%–77%.^{15,16,36} As *bla*_{CphA} is an MBL located in the bacterial chromosome, it reduces the risk of horizontal transmission of resistance genes compared to plasmid-mediated genes.³⁷ Except *bla*_{CphA}, we also detected nine strains of *Aeromonas* carrying *bla*_{KPC-2}-encoding gene (three of which contained both *bla*_{KPC-2} and *bla*_{CphA}) and two strains carrying *bla*_{NDM-1}-encoding gene. *Bla*_{KPC-2} and *bla*_{NDM-1} are the two major carbapenemases genes in *Enterobacteriaceae*. These two genes are usually located on plasmids and easily spread between different bacteria. The plasmid-mediated horizontal transmission of carbapenem resistance genes is the main reason for the global prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE).^{3,6} We speculate that these two carbapenem-resistant genes of *bla*_{KPC-2} and *bla*_{NDM-1} in *Aeromonas* may originate from *Enterobacteriaceae*. However, so far, the specific source and transmission mechanism remains to be determined.

As an opportunistic pathogen, *Aeromonas* can cause a variety of infections. Effective treatment of *Aeromonas* infection has become an urgent issue to be faced. Quinolones and the third- or fourth-generation cephalosporins could be used as the favorite antibiotics to empirically treat *Aeromonas* infection.^{38,39} Despite the fact that carbapenems can be effective drugs in the treatment of

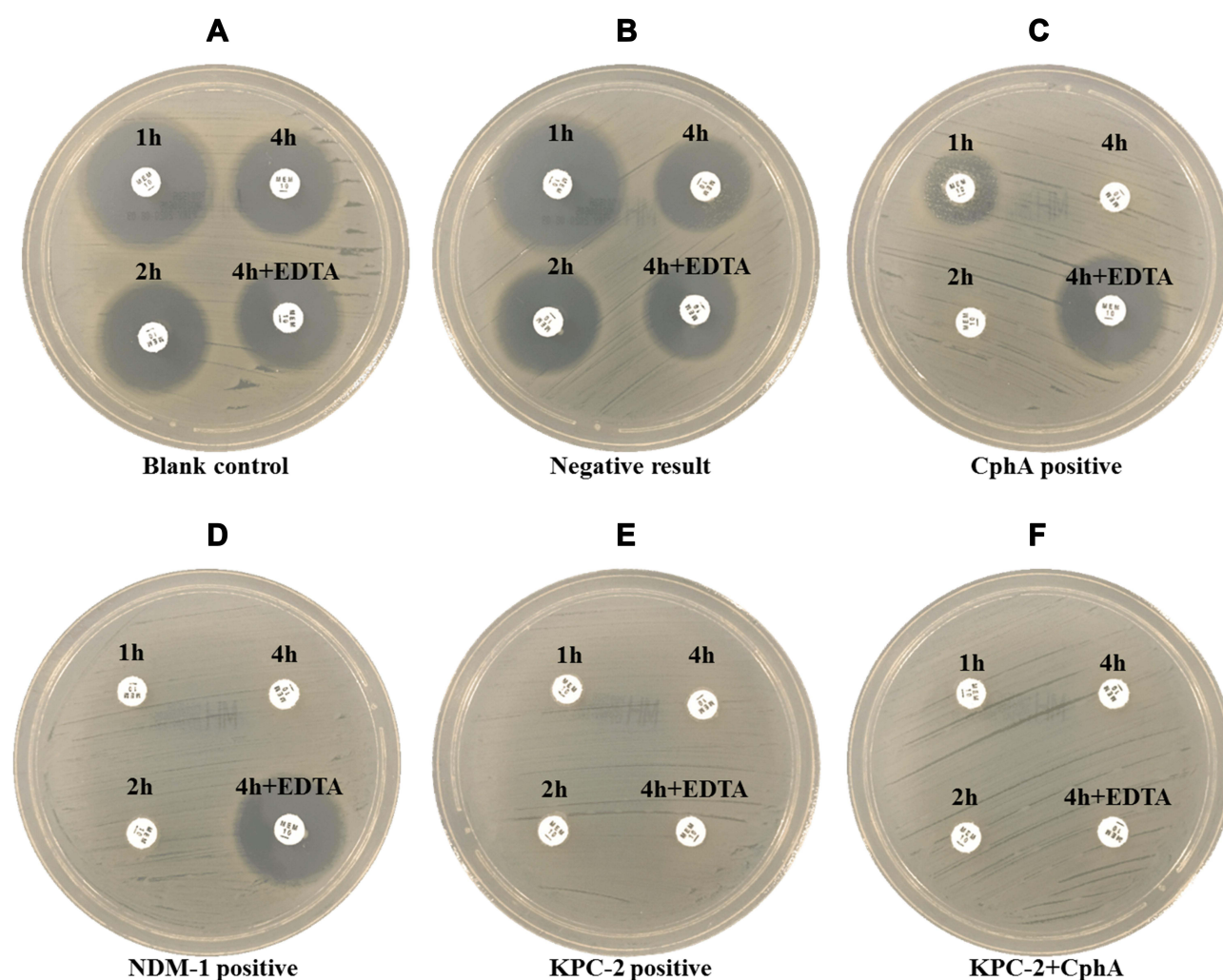


Figure 3 The representative photograph of CIM-s results in *Aeromonas*. The meropenem disks were immersed in 2mL supernatant or supernatant with 0.5M EDTA and incubated for 1h, 2h and 4h respectively. **(A)** Blank control. **(B)** Negative result. **(C–F)** Positive results, CphA **(C)** NDM-I **(D)**, KPC-2 **(E)** or KPC-2 + CphA **(F)**.

Abbreviation: CIM-s, carbapenem inactivation method using supernatant.

Gram-negative bacterial infection, they should be used prudently for *Aeromonas*. CphA, high prevalent in *Aeromonas*, has substrate specificity for carbapenem antibiotics, which can hydrolyze carbapenems, resulting in treatment failure. Moreover, for the *bla*_{CphA}-positive strains, the routine antibiotic susceptibility testing could not completely detect the carbapenem resistance phenotype, which had a high false-negative rate.^{15,16,40} Our results are consistent with previous studies. In 79 carbapenemase producers, the result of imipenem testing by VITEK2 was the best indicator with only 6 *bla*_{CphA}-positive strains not detected as resistant. The resistance rates of imipenem and meropenem, tested by disk diffusion or broth microdilution, were significantly reduced, and only by increasing the inoculum size could the resistance results be shown.^{15,40} The possible reason is that with standard

inoculum, it cannot express enough CphA to inactivate carbapenem, or there might be gene modification to influence the expression and activity of CphA. The specific mechanism needs further study. Therefore, routine disk diffusion or broth microdilution is not very suitable for conventional application. It is very important to find a simple and effective method to identify carbapenem resistance phenotype in *Aeromonas*.

Various phenotypic tests have been used in carbapenemase-producing Gram-negative bacilli with the advantages of user-friendly, affordable, accurate, and feasible for implementation in clinical microbiological laboratories.^{4,27} The application of phenotypic test in *Aeromonas* may be an ideal method to identify carbapenemase producer. Some researchers have found that MHT and Carba NP could detect carbapenemase activity in 97% of *bla*_{CphA}-positive

isolates.^{15,16} However, MHT and Carba NP test cannot distinguish specific carbapenemase types. Moreover, the above studies only tested the efficacy for CphA, and it was not known whether other types of carbapenemases in *Aeromonas* could be detected. mCIM, based on the growth test in the presence of carbapenem antibiotics,⁴ is a phenotypic screening test recommended by CLSI to detect CRE with high specificity and sensitivity. mCIM in combination with eCIM can distinguish serine carbapenemase and MBL except when co-produced serine carbapenemase and MBL organism that will emerge false negative of eCIM. Our results were consistent with the above description. In view of the high prevalence of *bla*_{CphA} in *Aeromonas*, it cannot be ruled out whether CphA MBL is co-produced, when the phenotypic test is interpreted as serine carbapenemase production. In our study, the sensitivity and specificity of mCIM for *Aeromonas* were both 100%, which were better than MHT and Carba NP test. The volume of bacteria used in *Enterobacterales* and *Pseudomonas aeruginosa* by mCIM is different. Therefore, we used 1- μ l and 10- μ l loopfuls of bacteria to carry out mCIM and eCIM in *Aeromonas* at the same time. The two results were completely consistent, so the 1- μ l was considered sufficient.

sCIM is another phenotypic test for the detection of carbapenemase based on mCIM with improvement of experimental technique.²⁸ In the sCIM, the organism is directly smeared onto an imipenem disk. The carbapenemases-producing isolate released carbapenemases to hydrolyze imipenem immediately, causing a decrease in the zone diameter. sCIM seems to be an effective method to identify the carbapenemase-producing isolates in the Gram-negative bacilli.²⁸ We applied this method to *Aeromonas*. Whereas for *bla*_{CphA}-positive isolates, there were six results interpreted as carbapenemase indeterminate result by sCIM. We speculate that the expression levels of CphA carbapenemase were different in *Aeromonas*, and the hydrolytic activity of CphA was weaker than KPC-2 and NDM-1. These strains could not effectively inactivate imipenem to reduce the inhibition zones. For those isolates of *bla*_{CphA} positive with sCIM indeterminate results, the results of mCIM were all positive. The cultural environment of the two methods was distinct. In mCIM, the organisms were grown in liquid TSB medium, which might be more conducive to release of CphA carbapenemase to effectively inactivate the carbapenem antibiotics. In sCIM, it was not beneficial to standardize as the bacteria to be tested were directly smeared onto an imipenem disk. The smeared bacteria

quantity will be different by distinct operators, and the concentration of bacteria on antibiotic disk could affect the size of zone.²⁸ Compared with sCIM, which cannot completely detect the *Aeromonas* harboring *bla*_{CphA}, mCIM is a more suitable method to identify and detect the carbapenemase in *Aeromonas*.

To further verify the hydrolytic activity of carbapenemase, especially CphA, secreted into the medium, we made further improvement on the basis of mCIM test, called CIM-s. We used the supernatant of bacteria cultured overnight in TSB broth, instead of bacterial suspension, to inactivate meropenem disk, and observed the change of inhibition zone in *E. coli* ATCC 25922 plates. We found that the supernatant containing KPC-2 and NDM-1 showed positive results after inactivation for 1 h. However, the positive rate of CphA supernatant was only 36.8% in 1 h. As the inactivation time extended to 4 h, the positive rate reached 97.1%. Only two strains showed indeterminate results. The carbapenem hydrolytic activity of KPC-2 and NDM-1 appeared significantly higher than that of CphA. By observing the inactivation of meropenem in the supernatant of *bla*_{CphA}-positive strains in CIM-s, it was confirmed that the expression and secretion ability of CphA was different in *Aeromonas*. There were strains with weak expression of CphA. After adding EDTA, the activity of NDM-1 and CphA could be inhibited, while KPC-2 was not affected. Therefore, the carbapenemase type could be preliminarily distinguished by CIM-s in combination with eCIM-s. Like eCIM, isolates containing both serine carbapenemase and MBL, such as KPC-2 and CphA, could not be detected accurately, only showing CIM-s positive.

By comparing mCIM, sCIM and CIM-s, the operation of sCIM is the most convenient as it does not require standard strains, TSB medium and extra incubation process. However, the sensitivity of sCIM is low, and sCIM cannot distinguish carbapenemase type. CIM-s needs a longer time and complex operation, which is not an ideal method to detect the carbapenemase, but it can be confirmed that the carbapenemase is secreted into the medium to exhibit hydrolytic activity. mCIM can detect a variety of carbapenemases in *Aeromonas*, including the weak expression of CphA, and the results are consistent with the gene detection. CphA is an inducible carbapenemase.^{41,42} After the addition of carbapenem antibiotics, the expression and secretion of CphA were promoted, and then effectively inactivate meropenem disk. For patients infected with *Aeromonas*, there is a high

amount of bacteria in the tissue, combined with the complex microenvironment, which may be conducive to the release of CphA, resulting in the continuous presence of CphA in the surrounding medium. The use of carbapenem drugs may further induce and promote the expression of CphA, leading to treatment failure.^{15,42} When it is considered to treat *Aeromonas* infection with carbapenems, it is recommended to perform mCIM combined with eCIM to detect whether it is a carbapenemase-producing strain.

Our study also had several limitations. All the isolates were from The Second Affiliated Hospital of Chongqing Medical University. The number of isolates to be verified was relatively insufficient. We did not identify *Aeromonas* to species level by housekeeping gene sequencing to compare interspecific differences. The carbapenemase gene profiles in this study were limited, only containing KPC-2, NDM-1 and CphA. A multicenter and large sample size verification is required to further confirm our results.

Conclusion

The infections caused by *Aeromonas* are complex and diverse. The use of carbapenem antibiotics should be cautious as the routine antibiotic susceptibility testing cannot detect carbapenem resistance phenotype completely for carbapenemase-producing *Aeromonas*. Our research expands the application of mCIM and eCIM. mCIM in combination with eCIM can effectively detect multiple carbapenemases in *Aeromonas*, which is suitable for carrying out in the microbiological laboratory. CIM-s reveals that these carbapenemases are secreted into the medium to perform their hydrolytic activities. In consideration of the high prevalence of carbapenemase, especially CphA, it is suggested to routinely apply mCIM and eCIM in *Aeromonas* as a supplement to the results of antibiotic susceptibility testing.

Ethical Approval

The clinical samples were part of the routine hospital laboratory procedure, not specifically isolated for this study, so an institutional review board was not required.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the

version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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