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Comparative Analysis of Three Phenotypic Methods to Determine Metallo-β-lactamase Production in Carbapenemase-producing Enterobacterales and *Pseudomonas aeruginosa* Isolates

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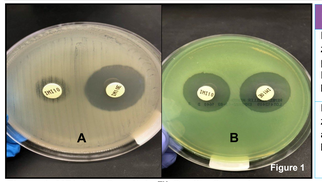
Introduction

- Carbapenemase producing organisms (CPOs) are an urgent antimicrobial resistance (AR) threat according to a 2019 CDC report and are a serious concern for patients in healthcare facilities.
- Carbapenemases are members of Ambler class A, B and D β-lactamases, with the ability to hydrolyze β-lactams including penicillins, cephalosporins and carbapenems.
- Metallo-β-lactamases (MBL) are class B carbapenemases and include NDM, VIM, IMP, SIM, GIM and SPM, among other gene families. MBLs require metal (zinc) for activity, therefore chelators such as EDTA can be used to inhibit the enzyme.
- Identification of CPOs and differentiation of MBLs from serine carbapenemases is important for infection control and therapeutic purposes.
- Current PCR methods generally target the "Big Five" carbapenemase families (KPC, NDM, VIM, OXA-48, IMP) but do not detect novel MBLs. However, novel carbapenemases can be detected by phenotypic methods.
- To enhance the detection of novel carbapenemases, Wadsworth Center (WC) added phenotypic methods for MBL production to the testing algorithm since phenotypic MBL tests are easy to use and less expensive than genotypic tests.
- WC evaluated three phenotypic methods for MBL production:
  1. MBL Neo-Sensitabs™ (Rosco Diagnostica)
  2. EDTA-modified Carbapenem Inactivation Method (eCIM)
  3. Imipenem / Imipenem + EDTA and Meropenem / Meropenem + EDTA Etests (MBL Etests, bioMérieux)

MBL Neo-Sensitabs™



Principle	Results & Interpretation	
If the test isolate is positive for MBL production, a large zone of inhibition will be present around the Imipenem 10 µg + EDTA 750 µg (IM10E) disk compared to the Imipenem 10 µg (IM10) disk, with a significant difference between the zone sizes.	MBL Positive	≥7 mm difference of IM10E from IMI10
If the test isolate is negative for MBL production, a large zone of inhibition will be present around both the IMI10 and IM10E disks, with no or a marginal difference between the zone sizes.	MBL Negative	<7 mm difference of IM10E from IMI10

Figure 1: MBL Neo-Sensitabs™ Quality Control
A. Positive Control: *Klebsiella pneumoniae* ATCC BAA-2146 (NDM-1), Calculation: IM10E (30 mm) – IMI10 (9 mm) = 21 mm
B. Negative Control: *Pseudomonas aeruginosa* ATCC 27853, Calculation: IM10E (26 mm) – IMI10 (26 mm) = 0 mm

Figure 2: MBL Neo-Sensitabs™ Materials and Methods
A. Using a nephelometer, a 0.5 McFarland (McF) isolate suspension is made in saline.
B. The isolate suspension is inoculated onto Mueller Hinton Agar (MHA).
C. The MBL Neo-Sensitabs™ disks, IMI10 and IM10E, are placed on the MHA, a minimum of 24 mm apart.
D. The MHA is placed in a 37°C incubator for 18-24 hours.

EDTA-modified Carbapenem Inactivation Method (eCIM)

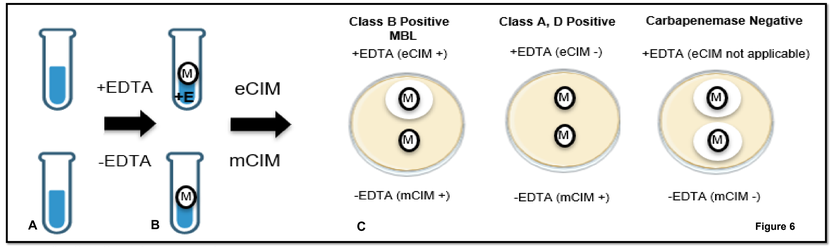
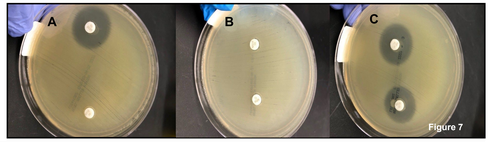


Figure 6: mCIM/eCIM Materials and Methods
A. mCIM and eCIM tests are performed in parallel by inoculating two Trypticase Soy Broths (TSB), one TSB containing 20 µl of 0.5M EDTA (eCIM). Inoculate with a 1 µl loop for Enterobacterales and a 10 µl loop for *Pseudomonas aeruginosa*. (*The eCIM is indicated for Enterobacterales only).
B. A 10 µg meropenem disk (M) is added to each TSB and incubated for 4 hours at 35°C.
C. Following incubation, a 0.5 McF suspension of *Escherichia coli* ATCC 25922 is prepared and inoculated onto MHA. The meropenem disks from both TSBs are placed on the MHA and incubated overnight at 35°C. Zones of inhibition are measured in mm and the difference in zone size is calculated. Three possible outcomes are pictured.

Principle	Results & Interpretation		
	mCIM Result	eCIM Result	Interpretation
If the test isolate is positive for MBL production, the activity of the MBL will be inhibited in the presence of EDTA such that the meropenem in the disk will not be hydrolyzed as efficiently as in the tube without EDTA. The result is inhibition of the meropenem-susceptible *E. coli* and an increase in the zone diameter for the eCIM zone diameter compared with the mCIM zone diameter.	Carbapenemase Positive	MBL Positive (≥5 mm increase in zone diameter)	Metallo-β-lactamase detected (Class B)
If the test isolate is positive for a serine carbapenemase, the activity of the carbapenemase will not be affected by the presence of EDTA and there will be no or marginal increase in zone diameter in the presence of EDTA compared with the mCIM zone diameter.	Carbapenemase Positive	MBL Negative (≤4 mm increase in zone diameter)	Serine carbapenemase detected (Class A, D)
eCIM is valid only if the mCIM is Positive	Negative	Do not interpret	Carbapenemase not detected
eCIM is valid only if the mCIM is Positive	Indeterminate	Do not interpret	Testing inconclusive for the presence of carbapenemase

Figure 7: eCIM Quality Control

A. Positive Control: *Klebsiella pneumoniae* ATCC BAA-2146 (NDM-1), Calculation: eCIM (21 mm) – mCIM (6 mm) = 15 mm
B. Positive Control: *Klebsiella pneumoniae* ATCC BAA-1705 (KPC), Calculation: eCIM (6 mm) – mCIM (6 mm) = 0 mm
C. Negative Control: *Klebsiella pneumoniae* ATCC BAA-1706, eCIM (do not interpret), mCIM (22 mm)



Imipenem / Imipenem + EDTA and Meropenem / Meropenem + EDTA Etests for MBL

Principle	Results & Interpretation	
If the test isolate is positive for MBL production, a larger ellipse will be present around the Imipenem 1 – 64 µg/mL + EDTA (IPI), or Meropenem 0.032 – 2 µg/mL + EDTA (MPI) end of the Etest compared to the Imipenem 4 – 256 µg/mL (IP) or Meropenem 0.125 – 8 µg/mL (MP) end of the Etest, resulting in a significant ratio.	MBL Positive	• Ratio (IP/IPI or MP/MPI) ≥8 µg/mL • Phantom zone • Deformation of ellipse
If the test isolate is negative for carbapenemase production, large ellipses will be present at both ends of the Etest, resulting in a small ratio.	MBL Negative	• Ratio (IP/IPI or MP/MPI) <8 µg/mL • Ratio (IP/IPI) 64 µg/mL / >64 µg/mL • Ratio (MP/MPI) <0.125 µg/mL / <0.032 µg/mL
See Figure 4. D and Figure 5. H.	MBL Non-determinable	• Both IP/IPI values are above or below the test ranges (≥256 µg/mL / ≥64 µg/mL or <4 µg/mL / <1 µg/mL) • Both MP/MPI values are above the test ranges (>8 µg/mL / >2 µg/mL)

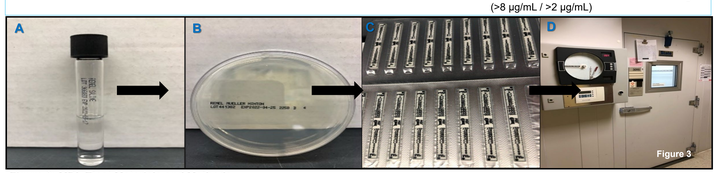


Figure 3: MBL Etest Materials and Methods
A. Using a nephelometer, a 0.5 McF isolate suspension is made in saline.
B. The isolate suspension is inoculated onto MHA.
C. The MBL Etest is placed onto the MHA.
D. The MHA is placed in a 37°C incubator for 16-20 hours.

Figure 4: IP/IPI Quality Control and Test Isolates (µg/mL)

A. Positive Control: *Stenotrophomonas maltophilia* ATCC 13636, Calculation: IP (>256) / IPI (3) = 85.33
B. Negative Control: *Pseudomonas aeruginosa* ATCC 27853, Calculation: IP (<4) / IPI (1.0) = 4
C. AR-Bank #0154: *Enterobacter cloacae* (VIM-1), Calculation: IP (<4) / IPI (<1.0) = Non-determinable, however a phantom zone is present = Positive
D. AR-Bank #0135: *Klebsiella pneumoniae* (VIM-1), Calculation: IP (<4) / IPI (<1.0) = Non-determinable

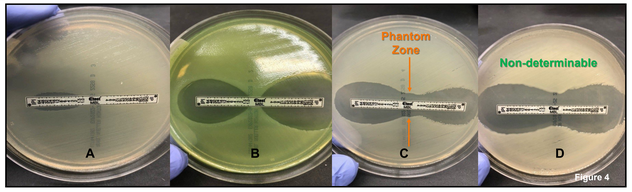
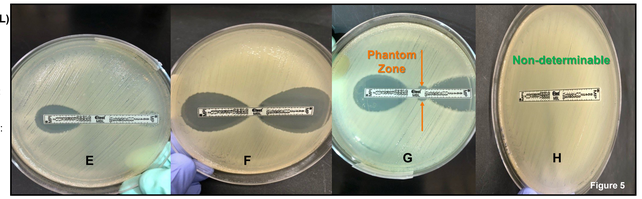


Figure 5: MP/MPI Quality Control and Test Isolates (µg/mL)

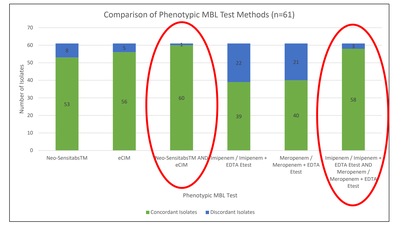
E. Positive Control: *Klebsiella pneumoniae* ATCC BAA-2146 (NDM-1), Calculation: MP (>8) / MPI (0.25) = 32
F. Negative Control: *Klebsiella pneumoniae* ATCC 700603, Calculation: MP (<0.125) / MPI (0.032) = 3.91
G. AR-Bank #0135: *Klebsiella pneumoniae* (VIM-1), Calculation: MP (0.25) / MPI (0.032) = 7.81, however, a phantom zone is present = Positive
H. AR-Bank #0138: *Klebsiella pneumoniae* (NDM-7),Calculation: MP (>8) / MPI (>2) = Non-determinable



Data

Positive MBL Isolates (n=43)	Total	Enterobacterales	*Pseudomonas aeruginosa*
NDM	17	17	
VIM	8	5	3
IMP	10	7	3
SIM	1		1
SPM	1		1
NDM/OXA-48-like	4	4	
IMP/KPC	1	1	
VIM/KPC	1	1	
Negative MBL Isolates*	18	18	
Total	61	53	8

*Negative MBL Isolates include KPC, OXA-48-like, IMI, KPC/OXA-48-like



Overall Analysis by Test Type (n=61)	Rosco Diagnostica Neo-Sensitabs™	eCIM	Rosco Diagnostica Neo-Sensitabs™ AND eCIM	bioMerieux Imipenem/ Imipenem +EDTA Etest	bioMerieux Meropenem/ Meropenem +EDTA Etest	bioMerieux Imipenem/ Imipenem +EDTA Etest AND Meropenem/ Meropenem +EDTA Etest
Accuracy (%)	87	92	98	64	66	95
Sensitivity (%)	81	88	98	72	65	93
Specificity (%)	100	100	100	44	67	100

Neo-Sensitabs™		Variants DETECTED	Variants NOT DETECTED
Enterobacterales	bla_{NDM}	1, 1/OXA-232, 5, 5/OXA-232, 6, 7	
	bla_{VIM}	1, 5/KPC-4, 27	
	bla_{IMP}		4, 6/KPC-3, 27, 38
Pseudomonas aeruginosa	bla_{NDM}	2, 4	
	bla_{VIM}	1, 13, 14	
	bla_{IMP}	1	
	bla_{SIM}	1	
eCIM		**Variants DETECTED**	**Variants NOT DETECTED**
Enterobacterales	bla_{NDM}	1, 1/OXA-232, 5, 5/OXA-232, 6, 7	
	bla_{VIM}	1, 27	5/KPC-4*
	bla_{IMP}	4, 27, 38	6/KPC-3*
Pseudomonas aeruginosa	bla_{NDM}	2, 4	
	bla_{VIM}	1, 13, 14	
	bla_{IMP}		1
	bla_{SIM}		1

*eCIM limitation: If both a serine carbapenemase and an MBL are co-produced by one organism, differentiation between enzymes may not be possible and false-negative eCIM results may occur

Imipenem/Imipenem+EDTA Etest		Variants DETECTED	Variants NOT DETECTED
Enterobacterales	bla_{NDM}	1, 1/OXA-232, 5, 5/OXA-232, 6, 7	1, 5
	bla_{VIM}	1, 1/KPC-4, 27	1
	bla_{IMP}		4, 6/KPC-3, 27, 38
Pseudomonas aeruginosa	bla_{NDM}	2, 4	
	bla_{VIM}	1, 13, 14	
	bla_{IMP}	1	
	bla_{SIM}	1	
Meropenem/Meropenem+EDTA Etest		**Variants DETECTED**	**Variants NOT DETECTED**
Enterobacterales	bla_{NDM}	1, 5, 6	1/OXA-232, 5/OXA-232, 7
	bla_{VIM}	1, 1/KPC-4, 27	1
	bla_{IMP}	4, 27, 38	6/KPC-3, 27
Pseudomonas aeruginosa	bla_{NDM}		2, 4
	bla_{VIM}		1, 13, 14
	bla_{IMP}		1
	bla_{SIM}		1

Imipenem/Imipenem+EDTA AND Meropenem/Meropenem+EDTA Etests		Variants DETECTED	Variants NOT DETECTED
Enterobacterales	bla_{NDM}	1, 1/OXA-232, 5, 5/OXA-232, 6, 7	
	bla_{VIM}	1, 1/KPC-4, 27	
	bla_{IMP}	4, 27, 38	6/KPC-3, 27
Pseudomonas aeruginosa	bla_{NDM}	2, 4	
	bla_{VIM}	1, 13, 14	
	bla_{IMP}	1	
	bla_{SIM}	1	
Neo-Sensitabs™ AND eCIM		**Variants DETECTED**	**Variants NOT DETECTED**
Enterobacterales	bla_{NDM}	1, 1/OXA-232, 5, 5/OXA-232, 6, 7	
	bla_{VIM}	1, 1/KPC-4, 27	
	bla_{IMP}	4, 27, 38	6/KPC-3
Pseudomonas aeruginosa	bla_{NDM}	2, 4	
	bla_{VIM}	1, 13, 14	
	bla_{IMP}	1	
	bla_{SIM}	1	

Results & Conclusion

- Of the 61 isolates analyzed, 43 were known MBLs, 11 were serine carbapenemases (KPC, OXA-48-like, IMI), and 7 were negative for carbapenemase production
- Percent accuracy was calculated for each test method; MBL Neo-Sensitabs™ 53/61 (87%), eCIM 56/61 (92%), MBL Neo-Sensitabs™ and eCIM used together 60/61 (98%), Imipenem / Imipenem + EDTA Etest 39/61 (64%), Meropenem / Meropenem + EDTA 40/61 (66%), and MBL Etests used together 58/61 (95%)
- Combined testing of the disk methods (Neo-Sensitabs™ and eCIM) or both MBL Etests demonstrated the best overall accuracy and sensitivity at >90%
- eCIM is indicated for Enterobacterales only, thus percent concordance for eCIM was calculated by organism type; Enterobacterales 50/53 (94%) and *P. aeruginosa* 6/8 (75%)
- The Neo-Sensitabs™ was not able to detect all IMP variants tested (IMP-4, IMP-27 or IMP-38), whereas the eCIM was able to detect these variants
- The Neo-Sensitabs™ was able to detect all dual mechanisms, with the exception of IMP-4/KPC-3, whereas the eCIM was not able to detect most dual mechanisms as it is a testing limitation
- The Neo-Sensitabs™ was able to detect the rare MBLs (SIM, SPM), whereas the eCIM was not
- MBL Neo-Sensitabs™ and eCIM were easy to implement and interpret
- MBL Etests were easy to implement, however interpretation was often difficult and complicated
- The WC Novel Carbapenemase testing algorithm was updated to incorporate both the MBL Neo-Sensitabs™ and eCIM to enhance detection of potential novel MBLs

Updated WC Potential Novel Algorithm

CRE/CRPA Potential Novel Isolates
(PCR for Big 5 negative / mCIM positive)

↓

- MALDI-TOF Identification
- PCR for Big 5 (KPC, NDM, OXA-48, VIM, IMP)

↓

mCIM / eCIM

↓

- Multiplex Novel PCR Panel (SIM, IMI, SPM)
- **Metallo-β-lactamase Production (Neo-Sensitabs™)**
- AST-GNX2F
- WGS

Acknowledgements

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