The laboratory identification of carbapenemases: an overview

Shamala Moodley [1] and Dharshni Pillay [2]

[1]Department of Biomedical Sciences, Mangosuthu University of Technology, Mangosuthu Highway, Umlazi, KwaZulu-Natal , South Africa.
[2]Department of Microbiology, University of Witwatersrand Academic Complex, National Health Laboratory Services; Department of Clinical Microbiology and Infectious Diseases, University of Witwatersrand, York Road, Parktown, Johannesburg, Gauteng, South Africa

Carbapenemase enzyme production is a newly-emerged antimicrobial resistance mechanism. Different enzymes have developed from the various β-lactamase families. (1) Early laboratory detection is of paramount importance. Early detection has a direct impact on antimicrobial therapy choices. (2) Additionally, infection control practices associated with transmissible resistance, are affected. (2) Phenotypic, genotypic and molecular testing methods are available. Each testing category plays a different role in the diagnostic cycle.

Keywords: carbapenem; carbapenemase; antimicrobial resistance; phenotypic testing; genotypic testing; molecular testing.

1. Introduction

Antimicrobial resistance predates the discovery of penicillin in 1928. (3) The modern "antibiotic era" produced unprecedented levels of resistance to nearly all antimicrobials and the emergence of multi-drug resistant "superbugs". (3) Normally susceptible organisms began to develop novel resistance mechanisms, such as methicillin resistant *Staphylococcus aureus* (MRSA). The burden of antimicrobial resistance was aggravated by transmissible resistance to susceptible organisms. (3)

2. Carbapenems

Carbapenems are a group of antimicrobials that bind penicillin-binding proteins (PBP) in the bacterial cell wall. (4) Cell wall formation is disrupted resulting in bacterial cell death. Many of the anti-microbial β-lactamase enzymes were unable to hydrolyse carbapenems. Therefore, carbapenems were originally used to treat infections caused by resistant microbes. (4) Subsequently carbapenem resistance mechanisms developed, notably the production of carbapenem hydrolysing enzymes (carbapenemase). (4, 5)

3. Carbapenemases

Carbapenemases are a group of β-lactamases that confer resistance to multiple β-lactam antibiotics, including penicillins, cephalosporins, β-lactam-inhibitor combinations and carbapenems. (6) Carbapenemases are commonly produced by gram negative organisms e.g. *Enterobacteriaceae* or *Acinetobacter baumannii*. (5) Their production can be either chromosomally-mediated (constitutive) or plasmid-mediated (acquired/transmissible). (6,7). Carbapenemases can be classified according to the Ambler classification of β-lactamases. (8) The Bush-Jacoby classification for β-lactamases may also be used. (1) However, this chapter will discuss the Ambler classification only. The Ambler system uses molecular characterization to categorised enzymes into classes based on distinguishing and conserved amino acid patterns. (9) Carbapenemases occupy Ambler classes A, B, and D (Table 1). (7,8) In response to antibiotic resistance, various strategies have been developed to identify these enzymes.

Table 1: Carbapenemase categorisation using the Ambler classification*.

Class	Active Site	Enzyme Family (Constitutive)	Enzyme Family (Acquired)
A	Serine	*Serratia marcesans* enzyme (SME) Non-metalloenzyme carbapenemase Imipenem-hydrolysing β-lactamase (IMI)	*Klebsiella pneumoniae* carbapenemase (KPC)
B	Zinc	Constitutive in certain organisms e.g. *Aeromonas* species (CphA)	Imipenemase (IMP) Verona-integron coded metallo-β-lactamase (VIM) New Dehli metallo-β-lactamase
C	Serine	*Not applicable to carbapenemases*	
D	Serine		Oxacillin-hydrolysing enzymes

**This table is not a comprehensive list of carbapenemase enzymes.*

4. Laboratory Methods Summary

Many laboratory methods have developed to assist with the identification of carbapenemase production. Methods are frequently used in combination.
Phenotypic test methods include (10):

1. Disk diffusion method (Kirby-Bauer method) - screening tests assisting in further laboratory and therapeutic decisions.
2. Minimum inhibitory concentration (MIC) determination – screening tests assisting in further laboratory and therapeutic decisions:
    - Broth microdilution method (BMD)
    - E-test method (bioMériuex, France)
    - Automated systems e.g. Vitek®2 (bioMériuex, France), MicroScan, Phoenix™ (BD, USA).
3. Modified Hodge Test: detects a variety carbapenemases, however, the exact enzyme cannot be accurately determined.
4. Double disc tests:
    - Ethylene diamine tetra-acetic acid (EDTA) - detects metallo-β-lactamase production.
    - 2-mercaptopropionic acid (MPA) - detects metallo-β-lactamase production.
    - Boronic acid: detects *Klebsiella pneumoniae* carbapenemase (KPC)-production.

Genotypic and molecular test methods (10):

1. Polymerase chain reaction: detects specific genes associated with various carbapenem enzymes.
2. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF): establishes the presence or absence of carbapenemase activity (qualitative test); new evidence to support enzyme detection.
3. Sequencing: single-molecule and whole-genome sequencing can be used to establish clonality of organisms during outbreaks or epidemiological surveillance.

5. Phenotypic Testing Methods

5.1 Antimicrobial Disc Diffusion Method

The disc diffusion method (Kirby-Bauer test method) may be used to screen for carbapenem resistance. (11) The test uses antibiotic-impregnated discs to test the response of an organism to specific antibiotics. (11) Antibiotics diffuse from the discs into the agar. The antibiotic concentration within the agar decreases with increasing distance from the disc. (12) The antibiotic MICs are inversely related to the sizes of the zones of inhibition. Therefore, an interpretation of the MIC can be made using standard curve graphs derived from zone sizes. (13) However, a formal MIC is needed to confirm carbapenem resistance. (12, 13)

A 0.5 McFarland standard suspension of the sample organism is made using normal saline. (14) The resulting solution is inoculated onto Mueller Hinton agar plates. Antibiotic discs such as imipenem, ertapenem, and meropenem are placed onto the plates. Antibiotic diffuses from the discs into the agar. The plates are incubated for 24 hours at 37°C. After incubation, zones of organism growth surrounding the various antibiotic discs are measured using a meter scale. (14) The results are interpreted using guidelines created by laboratory standards development institutes e.g. the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility and Testing (EUCAST). (15)

5.2 Minimal Inhibitory Concentrations:

MICs are the lowest concentration of a drug that will inhibit overnight bacterial growth. (16) Several testing strategies are available. All modalities of testing assist in establishing the presence of carbapenem resistance. (17) However, the carbapenemase activity cannot be differentiated from other mechanisms of carbapenem resistance. (18) Results may be confounded by carbapenemases that demonstrate susceptible, but raised, carbapenem MICs. (17, 18) Adjusted MICs from laboratory standards institutes could assist with this discrepancy.

- **Broth microdilution (BMD)** is the gold standard. (17) A microtitre plate is set using a series of broths mixed with serially diluted antibiotics solutions**. (**14) Each well on the plate contains each serial dilution of antibiotic i.e. a different concentration. After applying a standard inoculum to each well, the plates are incubated for 18-24 hours. Thereafter the MIC is read. The first well to show growth is the concentration value (MIC). (14)
- **Epsilometer tests (Etests)** show comparable results to BMD in many studies (10). It has proven to be a simple and accurate test. A test strip is infused with an antimicrobial gradient of approximately 1.5 MIC antibiotic dilutions. (14) A 0.5 McFarland standard suspension is made of the test stain in normal saline. The solution is swabbed onto the relevant plate (e.g. MHA) to create a confluent lawn. The E- test strips are placed onto the MHA plate and incubated at 37°C for 24 hours. Thereafter the zone of inhibition is recorded for the test strain (Figure 1). (14)
- **Automated techniques** such as Vitek®2 (bioMérieux, France), MicroScan and Phoenix™ (BD, USA) can determine MICs. In some studies, MicroScan showed comparable results to BMD. (10) However, these results may be based on similarity of the testing process between the two methodologies. Vitek 2 has been known to over-estimate MICs when compared to BMD. (10, 19) A confirmatory MIC on a different modality, may be needed in certain situations, such as in isolated ertapenem resistance. ([10, 20) KPC screening can be done using a combination of variables. (21) One study showed that screening based on automated carbapenem MICs were very sensitive e.g. MIC $\geq$ 2 (for meropenem on Vitek 2; a combination of ertapenem and imipenem/meropenem for BD Phoenix). (21)

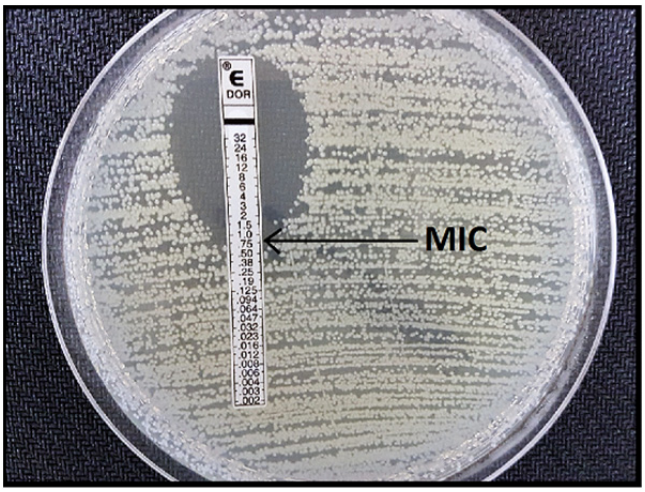

Figure 1: MIC by Etest on Mueller- Hinton agar. The MIC is the value at the junction of bacterial growth and the test strip.

5.3 Modified Hodge Test (MHT)

The MHT is a screening test for carbapenemase production. (22) The test allows for carbapenem inactivation by carbapenemase-producing bacteria. This allows a lawn of carbapenem susceptible bacteria to act as a growth indicator. If the test isolate produces the enzyme, the carbapenem is inactivated and allows growth of a susceptible carbapenem strain. (23) The carbapenem susceptible organism grows along the test organism growth streak. (24) A characteristic "clover leaf" indentation is created (Figure 2). (22-25) This test is sensitive for Class A and Class D enzymes. (25) However, metallo-β-lactamases (e.g. NDM-1) have consistently shown high false negative rates. (10, 25)

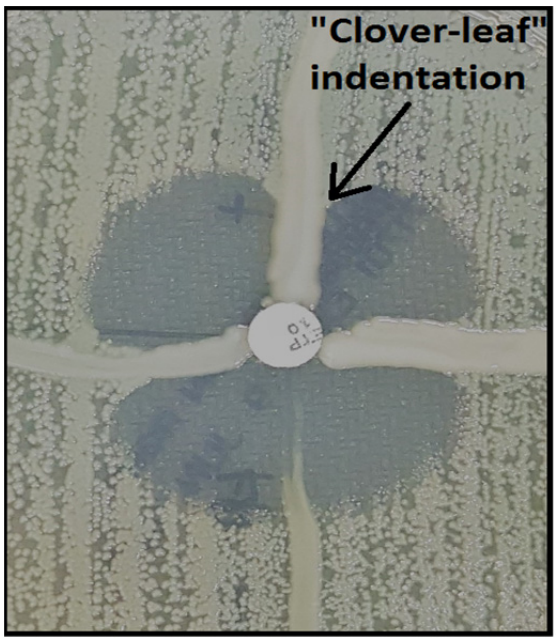

Figure 2: "Clover leaf"-like indentation on Mueller- Hinton agar.

The Centers for Disease Control and Prevention recommend the following technique. (22)

1. Use 300µl of 0.5 McFarland standard suspension of *E. coli* ATCC 25922 in 2700µl sterile normal saline as an indicator organism.
2. A 1:10 dilution mixture of this solution is streaked onto a MHA plate.
3. A 10µg meropenem or 10µg ertapenem disk is placed in the middle of the agar plate.
4. The test strain is streaked on the plate from the disc toward the periphery.
5. Positive (*K. pneumoniae* ATCC 1705) and negative controls (*K. pneumoniae* ATCC 1706) are streaked on to the same plate.
6. The plate is incubated at 37° for 24 hours.
7. Interpretation of the zones is done by viewing the clover leaf-like indentation of the *E. coli* 25922. (17)

The test is positive when the test strain produces carbapenemase which allows growth of the carbapenem susceptible *E. coli* ATCC 25922 strain towards the disk. A negative test is indicated by no growth of the isolate. (22)

5.4 Double Disc Tests

These screening tests require the addition of chelating agent to a carbapenem disc to enhance the effect of the antibiotic. (26) The tests should be used in combination with another screening test e.g. MHT. (27)

5.4.1 Metallo-β-lactamases (MBL)

MBL's display high level carbapenem resistance and require divalent zinc ions for hydrolysis. (26) Inactivation of the enzyme occurs through the application of chelating agents (Ethylene diamine tetra-acetic acid) or thiol compounds (2-mercaptopropionic acid).

- **Ethylene diamine tetra-acetic acid test (EDTA) test** is based on the zone enhancement around an imipenem disc when impregnated by EDTA. (26, 27) **EDTA** deprives MBLs of zinc. The resulting inactivation leads to detection of MBLs (Class B enzymes). (26)
  The technique incorporates disodium EDTA at a pH of 8.0. A 0.5 McFarland standard suspension of test strain in normal saline is swabbed on to a MHA plate. (14) Two imipenem discs are placed onto the plates. EDTA (4ul) is placed onto one disc. After 24 hours of incubation the inhibition zones around the imipenem and imipenem-EDTA discs are compared. A zone diameter difference between the two discs of ≥ 5 mm is positive for MBL production. (14)
- **2-mercaptopropionic acid (MPA)** is also an inhibition test that can detect MBL's. (26) The methodology of the test is similar to the EDTA test. Swab a 0.5 McFarland standard suspension of test strain (in normal saline) onto MHA plates. (14) Place 2 10µg imipenem discs 50mm apart on the plate. Add 3ul of MPA to one disc and incubate plates at 37°C for 24 hours. The diameters of growth inhibition are compared between the two

discs. Positive detection of MBL enzyme production is a difference of ≥5 mm between the inhibitory zones. (14)

5.4.2 KPC

KPC is a serine carbapenemase (Class A). (8) It can be detected using boronic acid. (28) Boronic acid is a Class C B-lactam inhibitor. (28,29) However, there have been proven effects against other serine classes. (30) According to Tsakris et al (2008), boronic acid detection of KPC showed higher sensitivity with meropenem discs compared to an ertapenem discs. (28) However, Asthana et al (2014) recommended ertapenem as the best screening antibiotic for KPC. (14) A testing method with 8 antibiotics is presented by Asthana et al (2014). (14)

Boronic acid test: Swab a 0.5 McFarland standard suspension of the test strain in normal saline onto MHC plates. (14) Place double discs of β-lactam antibiotics onto agar. The testing panel includes imipenem, meropenem, ertapenem, cefepime, cefoxitin, cefotetan, cefotaxime and ceftazidime. Add boronic acid to the second disc. Incubate plates at 37°C for 24 hours. Compare zone of inhibition between discs with and without boronic acid for the respective antibiotics. A positive test for KPC is a zone difference of ≥5 mm. (14)

6. Molecular/Genotypic Methods

6.1 Polymerase Chain Reaction (PCR)

PCR enables rapid detection of specific carbapenemase genes (Table 2). (31) Each gene corresponds to a specific enzyme. In addition, by using multiplex PCR, a range of enzymes are tested for simultaneously. (31) Clinical decisions and infection control targets are enhanced. (10) However, cost and the need for specialised expertise pose a disadvantage. (10)

DNA extraction is performed from bacterial colonies using DNA extraction kits. (31) Genes are detected with the relevant primers (Table 2). ([14)

A 50 µl reaction volume is utilised for PCR amplification of each gene target.

The reaction mixture contains:

- 10 mM Tris-Cl (pH 8.3)
- 50 mM KCl
- 2 mM $MgCl_2$
- 0.2 mM concentration of each dNTP
- 0.2 µM concentration of each primer
- 1.35U of Taq DNA polymerase. (14)

Initial denaturation is performed at 95°C for 10 min. (14) The PCR process occurs over approximately 35 cycles. Each cycle consists of:

- Denaturation at 94°C for 45 seconds
- Primer annealing at respective temperature (approximately 60°C) for 45 seconds
- Primer extension of DNA chain at 72°C for 50 seconds.

An additional extension step is performed for 7 min at 72°C. (14) Thereafter, the products are kept at 4°C. The products can be read using an agarose gel or fluorescent probes (in real-time PCR). *Escherichia coli* ATCC 25922 (negative control) and *Klebsiella pneumoniae* ATCC 700603 (positive control) should be run with every batch. (14)

6.2 Gene Sequencing

These methods can be used to detect specific genes, such as bla_{KPC} (KPC gene), bla_{NDM} (NDM-1 gene) or bla_{oxa24} (OXA-24 gene). (14) The products of PCR of the expected gene size are confirmed by sequencing methods. Once the PCR products are purified, an automated sequencer (e.g. ABI Prism 310; Applied Biosystems) processes the genetic material. (14) The resulting data can be analysed using a database or library e.g. BLAST. (32)

Table 2: Common Carbepenemase Gene Primers

Gene	Forward Primer	Reverse Primer	Base Pairs(bp)
bla_{KPC}	F-'CGGAACCATTCGCTAAACTC 3'	R-GGCGGCGTTATCACTGTATT 5'	107
Bla_{oxa23}	F-GATCGGATTGGAGGACCAGA 3'	R-ATTCTTGACCGCATTTCCAT 5'	501
bla_{oxa51}	F TAATGCTTTGATCGGCCTTG 3'	R–TGGATTGCACTTCATCTTGG 5'	353
bla_{oxa58}	F - TGGCACGCATTTAGACCG-3'	R- AAACCCACATACCAACCC 5'	507
bla_{oxa24}	F- TTCCCCTAACATGAATTTGT-3'	R-GTACTAATCAAAGTTGTGAA 5'	1024
bla_{VIM}	F- GATGGTGTTTGGTCGCATA 3'	R - CGA ATGCGCAGCACCAG 5'	390
bla_{IMP}	F-GAATAGAGTGGCTTAAYTCTC3'	R- CCA AACYACTASGTTATCT 5'	188
Bla_{NDM}	F-CACCTCATGTTTGAATTCGCC 3'	R- TCTGTCACATCGAAATCGC 5'	984

6.3 MALDI-TOF MS

Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) analyses molecules such as macromolecules, DNA, and proteins (33). MALDI-TOF MS in microbiology generates spectral graphs of an organism's protein content. (34) This spectral fingerprint is unique and can identify a specific organism. The technology can be applied to microbe identification and antimicrobial profile determination. (34-36)

Hydrolysis assays rely on the principle of carbapenem hydrolysis by carbapenemase enzymes present in a solution. (34) Incubation of the test organism in antibiotic solution results in antibiotic degradation, if carbapenemases production occurs. It is hypothesised that the mass change in a solution, resulting from hydrolysis of the antibiotic, is detectable by MALDI-TOF. Hydrolysed and non-hydrolysed states produce different spectral images. MALDI-TOF can be used qualitatively to establish the presence or absence of carbapenemase activity in enzyme-producing bacteria. (36)

Several testing methodologies have been developed. However, some basic principles apply. (34-36)

- Buffered solution of the antibiotic is mixed with bacterial culture.
- Incubation of this solution occurs.
- The incubated solution's supernatant is examined by the MS.

For example, the **imipenem hydrolysis assay,** as described by Gherbremendhi et al (2013) is as follows**. (**37)

- Inoculate a single colony of bacterial culture into 1ml sterile water.
- Centrifuge the solution at 13200 rotations per minute (rpm) for 1 minute.
- Resuspend the pellet into 20 mM Tris–HCl, 20 mM NaCl at a pH 7.0. A 3.0 McFarland standard is needed.
- Centrifuge 1mL of the solution at 13200 rpm for 1 minute.
- Resuspend the pellet into 50uL of reaction buffer with 0.1mM of imipenem.
- Incubate at 37°C for 4 hours in an Eppendorf Thermomixer.
- Centrifuge the solution at 13 200 rpm for 1 minute.
- The supernatant is used for MALDI-TOF analysis.

Specific carbapenemase enzymes may be detected. (34) One study demonstrated that NDM-1 or IMP-1 production was associated with complete ertapenem hydrolysis within 1 hour. KPC and VIM production was associated with a slower hydrolysis time-frame. (34)

7. Conclusion

Laboratory tests serve many functions in carbapenemase detection and characterisation. Therapy choice may be influenced by enzyme detection and specification. Susceptibility ranges also play a role in treatment choices. Clonality of organisms can be established during outbreaks or for epidemiological surveillance. (38) Infection prevention and control practices are affected if carbapenemase enzymes are present. (38) However, the key to all these doors rests in initial detection and laboratory confirmation.

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