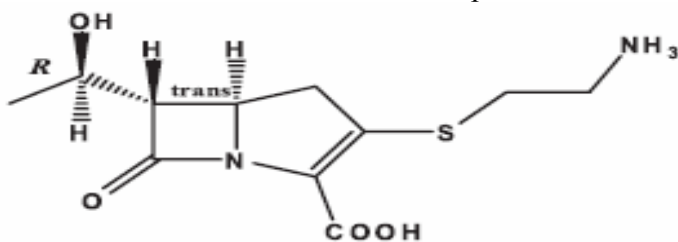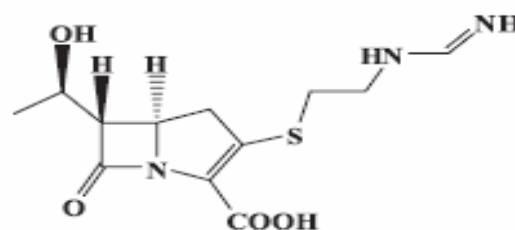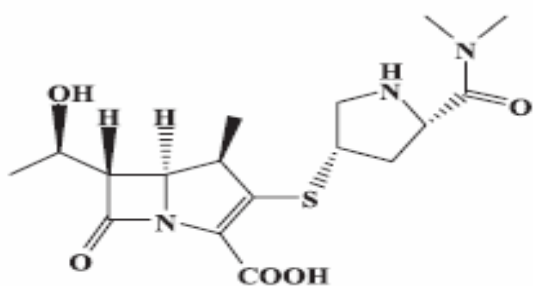# Carbapenemases

Carbapenems

Olivanic acids were the first natural products (*Streptomyces clavuligerus*) with beta-lactamase inhibitor properties to be identified. Because of poor penetration into bacteria and chemical instability, these compounds were not developed further. Thienamycin, a natural product from *Streptomyces cattleya* was identified as another beta-lactamase inhibitor (after clavulanic acid). It is the first "carbapenem" and served as a parent/model compound for all carbapenems. Carbapenem is a 4:5 fused lactam ring with a double bond between C2 and C3 but with substitution of carbon for sulfur at C1. The hydroxyethyl side chain is a key attribute of carbapenems and is important for its activity. Since thienamycin too was unstable in aqueous solution, more stable derivative such as Imipenem was developed in 1985. It was the first carbapenem to be used for treatment. Panipenem was developed subsequently; both of these drugs have high affinity to PBPs and stable against common beta-lactamases. However, both of these drugs are susceptible to hydrolysis by dehydropeptidase found in renal brush border. Hence, they have to be co-administered with the inhibitors such as cilastatin (or betamipron). Subsequently, carbapenems with better stability and broader spectrum such as meropenem, biapenem, doripenem and ertapenem were developed. Addition of methyl group to 1-β position was found to be protective against dehydropeptidase hydrolysis. Carbapenems (panipenem, meropenem, ertapenem, and doripenem) that have a pyrrolidine moiety as a side chain have broader antimicrobial spectrum.
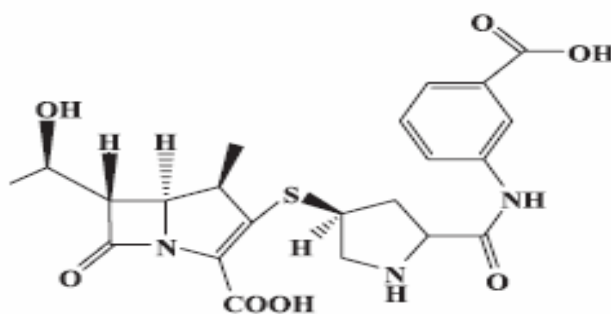
Carbapenems enter Gram-negative bacteria through outer membrane proteins (porins) and reach periplasmic space, where they permanently acylate the PBPs. Carbapenems, which have ability to bind to multiple different PBPs inhibit peptide cross linking and other peptidase reactions. Weakening of cell well and autolysis ensue resulting in death of the bacterium. Carbapenems have the broadest antimicrobial

spectrum than penicillin, cephalosporins or β-lactam/β-lactamase inhibitor combinations. In general, imipenem, panipenem, and doripenem are potent antibiotics against gram-positive bacteria whereas meropenem, biapenem, ertapenem (and doripenem) are slightly more effective against Gram-negative bacteria. Imipenem and meropenem have better activity than ertapenem against *P. aeruginosa*. Imipenem and doripenem have better activity than meropenem against *Acinetobacter baumannii*. Doripenem has lower MICs than do imipenem and meropenem against *P. aeruginosa* and *A. baumannii*. Hydrolysis of doripenem is several folds slower than that of imipenem and is the carbapenem that is least susceptible to hydrolysis by carbapenemases. Since all clinically available carbapenems have low oral bioavailability and thus do not cross gastrointestinal membranes readily, they must be administered intravenously. Imipenem-cilastatin and ertapenem can also be delivered intramuscularly. Carbapenems are used to treat serious infection and are often administered along with other antimicrobial agents. Excessive use of carbapenems can alter the intestinal microflora and select for carbapenem-resistant isolates.

Evolution of resistance:

Beta-lactams are naturally produced by some soil-dwelling bacteria (*Streptomyces sps*) or fungi in their natural environment. Other bacteria dwelling in the same environment have to protect themselves by producing enzymes that degrade these cell-wall acting substances produced by other microorganisms. During the course of evolution, some environmental bacteria (*B. cereus, B. anthracis*) started producing metallo-enzymes that provided them selective advantage for growth. These enzymes may also have some role in regulation of cell wall synthesis besides inhibition natural carbapenems (thienamycin, olivanic acids). The class A carbapenemase (SFC-1) was identified in an environmental isolate of *Serratia fonticola*. Many of the OXA carbapenemase genes have been identified in environmental isolates of *P. aeruginosa, A. baumannii* and *Shewanella spp*. In *A. baumannii*, the insertion sequences of ISAba1 type carrying strong promoters are present upstream of chromosomal OXA genes, resulting in increased carbapenemase expression. In these bacteria, OXA genes appear to be natural component of their respective chromosomes. Over times, these genes would have escaped from the chromosome into mobile genetic elements (integron, plasmids, transposons). Bacteriophage carrying OXA-type beta-lactamases gene have been recovered from the sewage, suggesting another vector for gene transfer among organisms.

Mechanism of resistance:

Mechanisms of resistance to carbapenems include production of β-lactamases, efflux pumps, and mutations that alter the expression and/or function of porins and PBPs. Combinations of these mechanisms can cause high levels of resistance to carbapenems in bacteria such as *K. pneumoniae, P. aeruginosa*, and *A. baumannii*. Carbapenem resistance in Gram-positive cocci is typically due to the result of substitutions in amino acid sequences of PBPs or acquisition/ production of a new carbapenem-resistant PBP. Carbapenem resistance in Gram negative bacteria is often due to production of β-lactamases, expression of efflux pumps, as well as porin loss and alterations in PBP. Production of β-lactamases appears to be the most widespread cause of carbapenem resistance. In addition to carbapenemases, resistances to carbapenems can also occur due to overproduction of certain AmpC beta-lactamases (CMY-10 and PDC [Pseudomonas-Derived Cephalosporinases]).

Carbapenem hydrolyzing enzymes:

Carbapenem hydrolyzing enzymes (also known as carbapenemases) are broadly divided into two types based on the reactive site of the enzymes; serine carbapenemases and metallo-β-lactamases. Carbapenemases represent the most versatile family of β-lactamases, which has unparalleled spectrum of substrates. Earlier carbapenemases were mostly species-specific and chromosomally encoded enzymes. Since 1990s, plasmid encoded carbapenemases have surfaced that have been observed in different organisms.

Classification:

Functional classification: The earlier method of classification of beta-lactamase enzyme involved biochemical analysis of enzyme, determination of isoelectric point, determination of substrate hydrolysis, enzyme kinetics and inhibition profiles. This functional classification of Karen Bush and others has undergone several revisions and currently divide beta-lactamases into four functional groups (1-3). The group 2 has several subgroups that are differentiated according to group-specific substrate or inhibitor profile. In this classification, carbapenemases fall under group 2f, 2df and 3.

Molecular classification: Ambler and others have classified beta-lactamases according to the amino acid sequences into four groups (A-D). Although this classification correlates well with the functional scheme, it lacks in the details concerning the enzymatic activity of the beta-lactamase. Molecular classes A and D contain beta-lactamases with serine in their active site while group B contains metallo-beta-lactamases with zinc in their active sites. Group B enzymes require one or two Zn^{2+} cations for activity and are subdivided into three groups, B1, B2, and B3, based on sequence alignments and structural analysis. Subclasses B1 and B3 have two zinc ions whereas subclass B2 has only one zinc ion. B2 enzymes are preferentially carbapenemases whereas B1 and B3 enzymes have broad hydrolysis spectrum.

Classification of carbapenemases:

Molecular class	Functional group	Enzyme	Hydrolysis profile					Inhibition profile	
			Penicillin	Early cephalosporin	Extended spectrum cephalosporin	Aztreonam	Carbapenems	EDTA	Clavulanic acid
A	2f	NMC	+	+	+	+	+	-	+
		IMI	+	+	+	+	+	-	+
		SME	+	+	±	+	+	-	+
		KPC	+	+	+	+	+	-	+
		GES	+	+	+	-	±	-	+
B1	3	IMP	+	+	+	-	+	+	-
		VIM	+	+	+	-	+	+	-
		GIM	+	+	+	-	+	+	-
		SPM	+	+	+	-	+	+	-
		NDM	+	+	+	±	+	+	-
D	2d	OXA	+	+	±	-	±	-	±

A. Molecular Class A enzymes:

Characteristic properties of these enzymes include presence of an active-site serine at position 70 and presence of a disulfide bond between Cys69 and Cys238 (changes the overall shape of the active site). The structural changes decrease the steric hindrance caused by the C-6 hydroxyethyl side chain of carbapenems, resulting in increased hydrolysis of imipenem. However, a single residue that is responsible for carbapenem resistance is still unknown.

All the enzymes have the ability to hydrolyze penicillins, early and extend-spectrum cephalosporins, aztreonam as wells as carbapenems. These enzymes are inhibited by clavulanic acid and tazobactam but not by EDTA. These enzymes are placed under functional 2f subgroup. GES-beta-lactamases were originally considered as ESBL but are now classified as serine carbapenemases. Early

class A carbapenemases were mostly chromosomal (SME, IMI, NMC) but recently plasmid mediated carbapenemases (KPC, GES) are on the rise.

Chromosomally encoded enzymes:

i.   **SME** (for *Serratia marcescens*): These enzymes were first discovered in *S. marcescens* isolate from England in 1982. There are currently only 3 SME types (SME-1, SME-2 and SME-3). These enzymes have been sporadically observed in *S. marcescens* isolates throughout the United States. No clonal spread among these isolates was observed.

ii. **IMI** (for imipenem hydrolyzing β-lactamase): This enzyme was first observed in an *Enterobacter cloacae* isolate in United States during 1984. Since then these have been rarely observed in clinical isolates of *E. cloacae* in United States, France and Argentina. Subsequently IMI-2 was reported from China, which was plasmid encoded.

iii. **NMC** (for not metalloenzyme carbapenemase): NCM-A enzyme was isolated from *E. cloacae* isolate in France during 1990 and subsequently reported from Argentina and United States.

NMC-A and IMI have 97% amino acid homology and are related to SME-1 with 70% amino acid homology. Even though these enzymes can hydrolyze extended spectrum cephalosporins, their rate of hydrolysis is comparatively less. Hydrolysis of cefoxitin too is inefficient. Cefoxitin and imipenem can induce these enzymes.

Plasmid encoded carbapenemases:

i.   **KPC** (for *Klebsiella pneumoniae* carbapenemase): this enzyme was first observed in a *K. pneumoniae* isolate from US in 1996. The resistance gene was associated with a large plasmid. Currently there are 12 known KPC enzymes. Although predominantly seen in *K. pneumoniae* isolates, they have been observed in *Salmonella enterica, K. oxytoca, E. cloacae, E. coli*, and *P. aeruginosa*. These enzymes confer resistance to all penicillins, cephalosporins, aztreonam and imipenem but remain susceptible to inhibition by clavulanic acid. The hydrolysis rates for imipenem, meropenem, cefotaxime and aztreonam are 10-fold lesser than those for penicillins and early cephalosporins. Even though these enzymes can hydrolyze carbapenems, the resistance is not apparent and in many cases the MIC values are less than the MIC breakpoints. This has resulted in under-detection of several KPC producers.

ii. **GES** (for Guiana extended spectrum): this enzyme was first observed in a *K. pneumoniae* isolate from French Guiana in 2000. The enzymes of the GES family differ from each other by 1-4 amino acid substitutions. Two enzymes with similar profile that were described as IBC-1 and IBC-2 (for integron borne cephalosporinase) have now been renamed as GES-7 and GES-8 respectively. The genes encoding GES family of enzymes are located in integrons on plasmids. In one *E. coli* isolate, GES gene (blaGES-7) is chromosomally located, and is the only *blaGES* gene that has not been identified on an integron. Because of their ability to hydrolyze extended spectrum cephalosporins they were initially considered to be ESBLs. Currently there are 22 known GES types. These have been observed in *K. pneumoniae, E. coli* and *P. aeruginosa* isolates from several nations (Greece, France, Portugal, South Africa, French Guiana, Argentina, Japan and Korea).

B. Molecular Class B enzymes:

These are metallo-β-lactamases (MBLs), which are characterized by its resistance to all penicillins, cephalosporins, beta-lactamase inhibitors, and carbapenems but are susceptible to inhibition by aztreonam and metal ion chelators (EDTA). Inhibition by EDTA can be reversed by adding Zn^{2+} ions. These

enzymes contains at least one Zn^{2+} ion in their active sites; B1 and B3 enzymes contain two Zn^{2+} ions where B2 enzymes contains only one Zn^{2+} ion. In fact, binding of another zinc ion decreases the activity of B2 enzymes. Zinc ions coordinate two water molecules that are necessary for hydrolysis. Zinc coordinating residues of B1 enzymes consists of two histidine and one cysteine. With class B2 enzymes, asparagine is found at the first position of the zinc binding motif HXHXD. Most MBLs have wide active site groove, which can accommodate most β-lactam antibiotics. Not all MBLs readily hydrolyze nitrocefin; MBL of an *Aeromonas sps* was identified not by nitrocefin assay but by carbapenem bioassay following isoelectric focusing. Most MBL genes (including VIM, IPM) are found as gene cassettes on class 1 integrons; few IMP genes are located on class 3 integrons. However, SPM-1 genes are not located on integrons or transposons. Mechanism of carbapenem hydrolysis is complex and varies from one MBL to another. Across the groups, MBLs share only 25% amino acid homology, but all share the same unique αββα fold.

Chromosome borne MBLs:

The first metallo-β-lactamases were detected in environmental and opportunistic pathogenic bacteria (*Bacillus cereus* (BCI, BCII), *Aeromonas spp* (CphA) and *Stenotrophomonas maltophilia* (L1)) as chromosomally encoded enzymes. These bacteria also produced additional serine beta-lactamases and both the enzymes were inducible by exposure to β-lactams. Chromosomal MBL was also found in few strains of *Bacteroides fragilis* (CcrA).

Plasmid borne MBLs:

Plasmid mediated MBLs have now attained significance because of its global spread. The majority of the mobile MBL genes are found as gene cassettes. These include blaIMP, *blaVIM, blaGIM, blaSIM,* and *blaKMH*.

IMP (for active on imipenem): Transferable carbapenem resistance was first detected in a *P. aeruginosa* isolate from Japan in 1990. They were subsequently reported in four *S. marcescens* isolates in Japan. This was followed by another report describing such a resistance in an isolate of *B. fragilis*. IMP-2 was observed in *A. baumannii* in Italy. Currently, there are 37 known IMP types. While these are more commonly seen in P. aeruginosa and A. baumannii isolates, they have been reported from most Enterobacteriaceae members.

VIM (for Verona integron encoded metallo-β-lactamase): This class 1 integron associated MBL was first observed in a *P. aeruginosa* isolate from Italy in 1997. It is most closely related in BCII with only 39% amino acid homology. VIM-2 was reported in a clinical isolate of *P. aeruginosa* from France. Currently there are 34 known VIM types. VIM-2 is the most dominant MBL across Europe. It has been detected in more than 23 species across 40 countries.

SPM-1 (for Sao Paulo metallo-β-lactamases) was identified in a *P. aeruginosa* isolate from Sao Paulo, Brazil. Genetic analysis has revealed that it is not a part of any integron but is associated with a new type of transposable structure. *blaSPM-1* is a part of mobile pathogenicity island located on a plasmid.

GIM-1 (for German imipenemase) was first isolated from Germany in 2002. GIM-1 had no clear preference for any substrate and did not hydrolyze azlocillin, aztreonam, and the serine-β-lactamase inhibitors. *blaGIM-1* was found on class 1 integron in a 22-kb nontransferable plasmid.

SIM-1 (for Seoul imipenemase) was first isolated from *P. aeruginosa* and *A. baumannii* isolates during a large scale screen of imipenem resistant isolates in Seoul. All SIM-1-producing isolates exhibited relatively low imipenem and meropenem MICs (8 to 16 µg/ml) and had a multidrug resistance phenotype.

NDM-1 (for New Delhi metallo-β-lactamase) was first reported in 2009 from a *K. pneumoniae* isolate obtained from a Swedish patient of Indian origin, who had received medical treatment in India. Located on a 180 kb plasmid, it expressed high level resistance to all penicillins, cephalosporins, aztreonam, cefoxitin, carbapenems and ciprofloxacin. It was susceptible only to colistin. PCR analysis failed to detect previously known MBL genes but did detect a class 1 integron. This isolate also harboured an AmpC (CMY-4) gene, a novel erythromycin esterase gene (*ereC*) as well as genes coding for resistance to chloramphenicol and aminoglycosides. NDM-1 shares very little identity with other MBLs, with the most similar MBLs being VIM-1/VIM-2, with which it has only 32.4% identity. Compared to VIM-2, NDM-1 displays tighter binding to most cephalosporins, in particular, cefuroxime, cefotaxime, and cephalothin, and also to the penicillins. However, NDM-1 does not bind to the carbapenems as tightly as IMP-1 or VIM-2. NDM-1 not only is a new subclass of the B1 group of MBLs but also possesses novel amino acids near the active site, suggesting that it has a novel structure. Even though class 1 integron was detected in the isolate, NDM gene was not carried in it or present near ISCR1 element. MLST determined that the *K. pneumoniae* belonged to ST14. Currently, there are six known NDM types. Since its detection, NDM-1 producing *E. coli* and *K. pneumoniae* have been detected in several parts of the world. To make matters worse, even colistin and tigecycline resistant NDM-1 producers have been found in India. A NDM-1 producing *Citrobacter freundii* isolate was found to harbour nine different beta-lactamase genes. Most other NDM-1 enzymes are susceptible to aztreonam, colistin and tigecycline. Co-production of other AmpC and ESBLs may hamper the use of aztreonam.

C. Molecular Class D enzymes:

Class D enzymes are OXA (for oxacillin hydrolyzing) enzymes, which are penicillinases capable of hydrolyzing oxacillin and cloxacillin. These serine beta-lactamases are plasmid encoded and are found commonly in Enterobacteriaceae and *P. aeruginosa*. These are poorly inhibited by clavulanic acid and EDTA and are known to have large amount of variability in amino acid sequences. Currently there are 239 OXA enzymes, of which at least 9 are ESBLs and at least 37 are carbapenemases. These are classified as subgroup 2df in the functional classification.

Cluster	Enzyme family	Additional members
1	OXA-23	OXA-27, OXA-49
2	OXA-24	OXA-25, OXA-26, OXA-40, OXA-72
3	OXA-51	OXA-64 to OXA-71, 75-78, 83, 84, 86-89, 91, 92, 94, 95
4	OXA-58	-
5	OXA-55	OXA-SHE
6	OXA-48	OXA-54, OXA-SAR2
7	OXA-50	OXA-50a- OXA-50d
8	OXA-60	OXA-60a - OXA-60d
9	OXA-62	-

The first OXA enzyme with carbapenemase activity was observed in an *A. baumannii* isolate from Scotland in 1985. It was originally named ARI-1 (for Acinetobacter resistant to imipenem). The enzyme was encoded on a large plasmid. Sequencing the enzyme revealed that it belonged to OXA family of beta-lactamases. The enzyme was later renamed as OXA-23. Most of these enzymes are encountered in *Acinterobacter spp*. Based on amino acid homologies, OXA carbapenemases are sub-divided into nine major subgroups (1-9).

OXA 23, 24 and 51 have been isolated from clinical isolates of *Acinetobacter spp*. OXA-50 has been observed as chromosomal enzymes in several P. aeruginosa strains; these are not expressed all the time and may not cause overt carbapenem resistance. Similarly OXA-51 has been found as chromosomal enzymes in several *A. baumannii* strains. OXA-48 was isolated from a clinical isolate of *K. pneumoniae*

from Turkey, which had <50% amino acid homology with other OXA enzymes. This plasmid borne enzyme has the highest rate of imipenem hydrolysis rate of all the OXA carbapenemases.

Among the carbapenem hydrolyzing OXA enzymes, there is 40-70% amino acid homology within groups and within the group the homology is 92.5% or higher. The catalytic mechanism of these enzymes is similar to that of serine carbapenemases. These enzymes have measurable activity against penicillins, early cephalosporins and imipenem. The extended spectrum cephalosporins are hydrolyzed poorly. Additionally, efflux pumps are also thought to contribute to carbapenem resistance.

Epidemiology:

International travel is an important risk factor for colonization or infection with antibiotic-resistant organisms; the risk being highest among travelers to India, the Middle East and Africa.

Genes coding for KPC carbapenemases are all coded on plasmid, but none the genes were found on gene cassettes. KPC has been reported in the United States, Israel, Turkey, China, India, and the United Kingdom. They have been observed in *K. pneumoniae, K. oxytoca, E. coli, E. cloacae, E. aerogenes, C. freundii, S. enterica, S. marcescens* and *P. aeruginosa*. KPC enzymes were originally discovered in US, within short time it was reported from several states across US. Several instances of outbreaks were reported from US hospitals during 2001-2003 that were caused by KPC-2 and KPC-3 producing *K. pneumoniae*. Patients from Israel and Columbia, from whom KPC-2 producing *K. pneumoniae* were isolated, did not give any history of travel to US; suggesting that they might have risen from a common but unknown ancestor. Since 2005, KPCs were detected in various species across the world.

Although rare, GES enzymes have been identified world wide including Greece, France, South Africa, Portugal, Brazil, Argentina, Korea and Japan. Most of these isolations were of single occurrences; however one clone of *P. aeruginosa* was associated with a small hospital outbreak in South Africa during 2000. Isolates producing GES enzymes with carbapenemase activity have been observed predominantly in Europe, South Africa and China.

Examples of the dissemination of organisms carrying carbapenemases include VIM-1 in Greece, Israel, Spain and Italy; and IMP-type carbapenemases in Asia and Brazil. While VIM and IMP enzymes continue to be detected all over the world, GIM, SIM and SPM enzymes have not spread beyond their countries of origin. VIM-2 is the most commonly isolated MBL from many parts of the world, but since 2009, reports of NDM MBL detections are on the rise. The gene encoding NDM-1 is located in a very mobile genetic element, resulting in rapid spread across the species and countries. The NDM-1 has spread from India, Pakistan and Bangladesh to UK, US, France, Kenya, Japan, Canada, Belgium, the Netherlands, Taiwan, Singapore, Sultanate of Oman, and Australia. A chief risk factor identified among patients infected with NDM-1–producing bacteria has been admission to hospitals in the Indian subcontinent. A substantial number of infected/colonized patients have been part of the so-called "medical tourism."

OXA-48 is mainly found in *K. pneumoniae* and that is now reported from Turkey, China, India, and the United Kingdom. OXA-24 and OXA-40 were reported from hospital outbreaks by *A. baumnnii* in Spain and Portugal. OXA-23 has been reported from hospital outbreaks by *Acinetobacter spp* in Brazil, UK and Korea. OXA-23 and OXA-58 have been recovered from infections in civil and military personnel serving in Iraq and Afghanistan. OXA-58 is routinely found in *Acinetobacter spp* isolates in France, Greece, Romania, Italy, Turkey, Kuwait and Argentina.

Laboratory detection of carbapenemases:

A resistant phenotype or decreased susceptibility to carbapenems indicates the possibility of carbapenemase production. With enzymes such as GES and KPC, the expression of carbapenemase resistance is weak, so that it often goes unnoticed and undetected in routine susceptibility testing. Screening the isolates against cephalosporins and carbapenems gives indication of carbapenemase presence. CLSI suggested that it better not to detect carbapenemases routinely but instead to reduce carbapenem breakpoints so that a susceptible test predicts effective therapy. It is not necessary to test an isolate for a carbapenemase by the MHT when all of the carbapenems that are reported by a laboratory test either intermediate or resistant. However, detection tests may be done for epidemiological and academic purposes.

1. Screening tests:

a. Screen agars:

Several commercial chromogenic media for screening of carbapenem-resistant Enterobacteriaceae have also been recently introduced and include CHROMagar[TM] KPC, Brilliance[TM] CRE and Hardy CHROM[TM] Carbapenemase.

b. Susceptibility breakpoints:

The susceptibility breakpoints recommended by CLSI (2011; M100-S21)

Carbapenem	Disc diffusion breakpoints (zone diameters in mm)			MIC breakpoints (µg/ml)		
	R	I	S	R	I	S
Imipenem10µg	≥23	20-22	≤19	≤1	2	≥4
Meropenem 10µg	≥23	20-22	≤19	≤1	2	≥4
Ertapenem 10µg	≥23	20-22	≤19	≤0.25	0.5	≥1
Doripenem 10µg	≥23	20-22	≤19	≤1	2	≥4

CLSI recommends that zone diameter of 16–21 mm for ertapenem and 14–21 mm for meropenem may be considered indicative of carbapenemase production despite the fact that they are in the current susceptible interpretive categories. Similarly, a MIC of 2-4 µg/ml of ertapenem and 2-8 µg/ml for imipenem and meropenem should indicate possible carbapenemase production.

Only meropenem and imipenem are recommended for testing against *P. aeruginosa* and *Acinetobacter spp*. Imipenem MICs for *Proteus spp*, *Providencia spp*, and *M. morganii* tend to be higher than meropenem or doripenem MICs. Since imipenem disc test performs poorly as a screen for carbapenemases, CLSI recommends using ertapenem or meropenem discs.

Most serine carbapenemases are identified by reduced or full susceptibility to extended-spectrum cephalosporins, aztreonam and carbapenems but inhibition by clavulanic acid and tazobactam. MBLs are identified by reduced susceptibility to extended spectrum cephalosporin and carbapenems but susceptibility to aztreonam and EDTA. This susceptibility is reversed by the addition of Mg^{2+}.

Ertapenem has better sensitivity over imipenem and meropenem in detecting KPC producing isolates. Disc diffusion lacks discrimination and MIC determination by microbroth dilution or agar dilution is preferred. Co-production of ESBL, AmpC beta-lactamases and porin mutants can distort the specificity of the test. MIC determination can be achieved by E-test strips and automated systems such as Microscan Walkway, Vitek, Vitek2, BD Pheonix Sensititre etc. The results produced by the automated systems are not consistent and E-test results are also sometimes inconsistent due to colonies in the zone of

inhibition. Increased carbapenem MICs in Enterobacteriaceae may also result from high expression of AmpC or CTX-M ESBLs in combination with porin alterations.

## 2. Phenotypic detection of carbapenemase activity

a. Modified Hodge test (MHT)

This test detects carbapenemase production in isolates of Enterobacteriaceae but does not differentiate between serine and metallo-beta-lactamases.
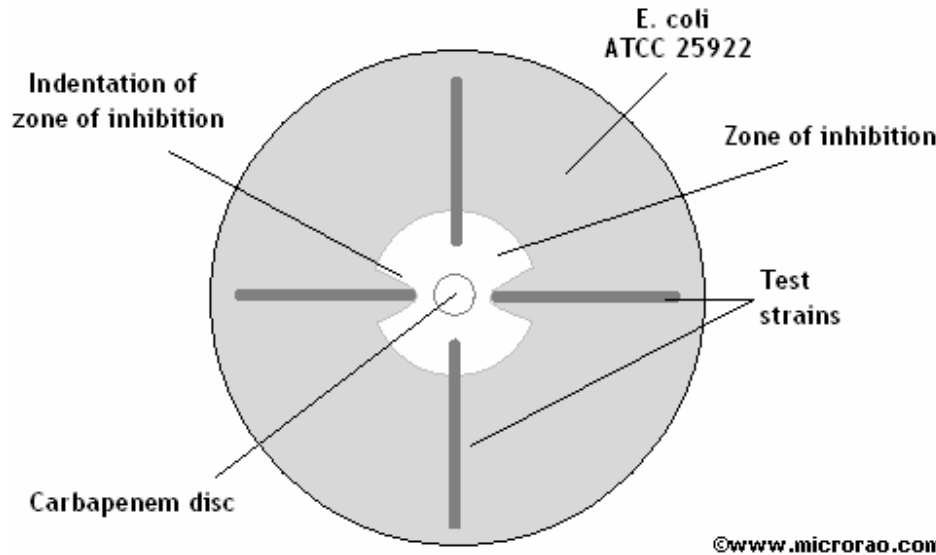
The test relies on formation of characteristic clover leaf like indentation in the zone of inhibition of a carbapenem susceptible strain (*E. coli* ATCC 25922) around a carbapenem disc due to carbapenemase produced by the test strain. The 1 in 10 dilution of 0.5 McFarland standard suspension of the susceptible strain is inoculated on Mueller Hinton agar so as to produce a semi-confluent growth. A carbapenem disc (imipenem or ertapenem 10µg) is placed in the center of the plate. The test organisms are streaked in a straight line from the edge of the disc to the edge of the plate. The streak should be at least 20 to 25 mm in length. Up to four isolates can be tested on the same plate. The plate is incubated in ambient air at 37°C for 18-24 hours. A clover leaf-type indentation at the intersection of the test organism and the E. coli 25922, within the zone of inhibition around the carbapenem disc is considered positive for carbapenemase production. Following quality control strains may be used with each run: MHT Positive *K. pneumoniae* ATCC BAA-1705, MHT Negative *K. pneumoniae* ATCC BAA-1706. The test can be modified slightly by testing eight isolates in a single but bigger plate (as shown in the diagram.). In this diagram isolates 2, 4, 6, and 7 are positive.
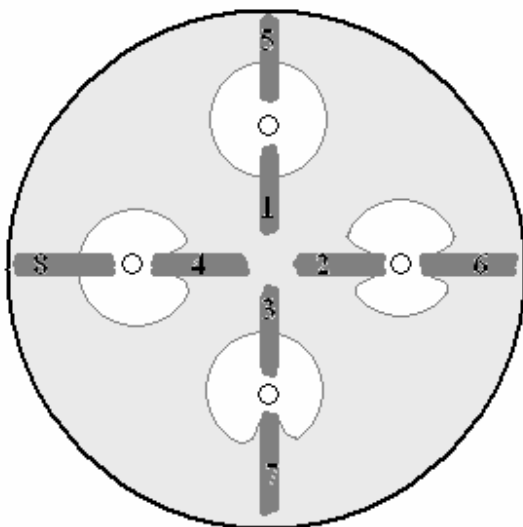

The performance of the Hodge test can be improved by addition of zinc sulfate (140µg/disc) to an imipenem disc. Advantage of this test is that enzymes that have very weak carbapenemase activity (OXA-23, GES-5, GES-6) can be detected by this method. The test may be false-negative as enzymes may not be sufficiently released to participate in the test since the MHT uses intact cells. Falsely positive results can occur with high-level AmpC producers, which more likely to occur with an imipenem disc. Further modification of modified Hodge test ('double-modified Hodge test') can be performed to avoid false-positive results of the modified Hodge test in isolates producing AmpC and/or ESBL with decreased permeability. The test may be performed with discs containing a carbapenem plus oxacillin to inhibit AmpC β-lactamases and with discs containing a carbapenem plus 3-aminophenylboronic acid to inhibit AmpC and class A carbapenemases.

b. Synergy test

Synergy test is based on in vitro inhibition of carbapenemase activity by addition of an inhibitor specific for a class of carbapenemases. For detection of class A carbapenemases, the inhibitor used is boronic acid (3-aminophenylboronic acid) and for detection of class B metallo-carbapenemases, ethylene diamine tetra-acetic acid (EDTA), 2-mercaptopropionic acid, sodium mercaptoacetic acid or dipicolinic acid can be used as an inhibitor.

Imipenem disc

Enhancement in the zone of inhibition

Test strain

EDTA disc
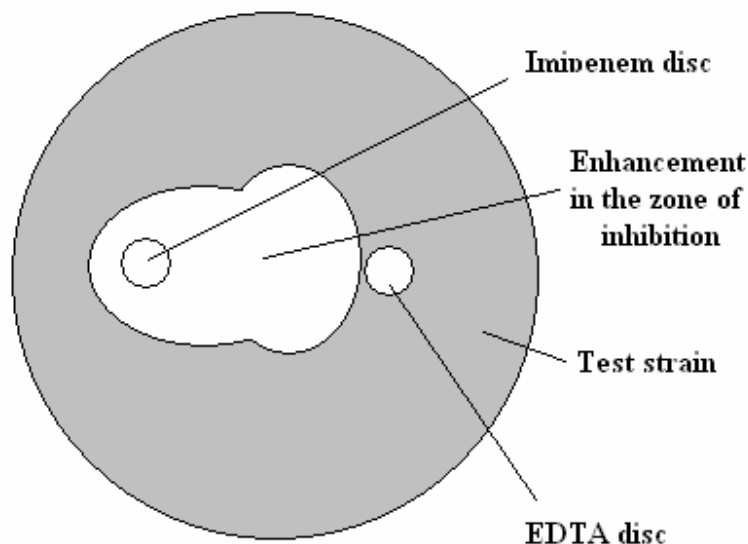
Double disc synergy test or disc approximation test has been often used to detect MBL producers. This relies on inhibition of MBL by EDTA. Following inoculation of the test isolate on Mueller Hinton agar plate to produce a semi-confluent growth, two discs are placed separated by a distance of 20-30 mm; one containing beta-lactam and the other containing EDTA. Enhancement of zone around the beta-lactam disc is considered positive for MBL production. Imipenem, ceftazidime and cefepime have been used as the beta-lactam drug for this test. Ceftazidime-mercaptopropionic acid, ceftazidime-mercaptoacetic acid, ceftazidime-clavulanate-EDTA and cefepime-clavulanate-EDTA too have been tried to detect MBLs. A study reported that imipenem-EDTA combination was most sensitive in detection of MBL in *P. aeruginosa* and *A. baumannii*, while ceftazidime-clavulanate with EDTA was most sensitive for *K. pneumoniae* and cefepime-clavulanate with EDTA was sensitive for *E. cloacae* and *C. freundii*. Sensitivity of the test depends on the optimal distance between the discs.

Combination disc methods have been used to more accurately identify the carbapenemase type. This method involves use of two discs; one with a carbapenem antibiotic and the other with carbapenem with an inhibitor. An increase in the zone diameter around the disc with inhibitor by 4-5mm or above over the carbapenem disc indicates positive carbapenemase production. For detection of Class A carbapenemases, imipenem (10µg) and imipenem (10µg) + 3-aminophenylboronic acid (400 µg) discs are placed over the inoculated Mueller Hinton agar. 3-aminophenylboronic acid has an inhibitory activity on KPC carbapenemases. When combined with phenyl boronic acid (PBA), ertapenem tends to exhibit some false-positive results in AmpC-producing strains; this can be avoided by using meropenem or imipenem. For detection of class B carbapenemases (MBL), combined disc method uses a carbapenem disc and a disc with carbapenem plus EDTA (10µL of 0.1 M). Another adaptation of this method uses four discs; meropenem (10µg), meropenem (10µg)+EDTA (10µL of 0.1 M), meropenem (10µg)+ and PBA (400 µg), meropenem (10µg)+EDTA (10µL of 0.1 M) + PBA (400 µg). EDTA may increase cell permeability and the released zinc may accelerate imipenem decomposition and decrease OprD expression in *P. aeruginosa*, thereby giving false positive result.

Meropenem	Meropenem + PBA	Meropenem + EDTA	Meropenem + PBA + EDTA	Interpretation
-	+	-	+	Class A carbapenemase
-	-	+	+	Class B carbapenemase
-	-	-	+	Both class A and B carbapenemase

Etest MBL strips for metallo-β-lactamase testing are available as imipenem and imipenem-EDTA combinations. The strip contains imipenem gradient (256-4 µg/ml) and imipenem+EDTA (64-1µg/ml + 320µg/ml). A threefold-or-greater decrease in the imipenem MIC in the presence of EDTA indicates MBL production. This method has good sensitivity and specificity but false-negative results have been reported when an isolate had an imipenem MIC of <4 µg/ml. Individual colonies within zone of inhibition makes interpretation difficult. False positive results can occur with some bacteria as EDTA alone has inhibitory action due to permeabilization of the outer membrane. Etest MBL detection tests have also yielded false-positive results with OXA-23-producing *A. baumannii*.

c. Detection of carbapenemase activity: The carbapenemase activities of cell sonicates from overnight broth cultures are determined by spectrophotometric assays. These are undertaken by using 150 µM imipenem as the substrate. The assays are performed with or without EDTA (25 mM) to examine the inhibition of carbapenemase activity. Measurement of hydrolysis is undertaken at 299 nm. This method does not significantly distinguish between the different carbapenemase types. Large amounts of extracts are used for isolates that produce very weak carbapenemases.

d. Estimation of isoelectric points: The sonicated extracts (containing the enzymes) of the cells are separated on basis of their isoelectric pH by isoelectric focusing. Beta-lactamase activity is identified by using chromogenic cephalosporin nitrocefin. Overlay of the gel with EDTA, clavulanic acid, or aztreonam can detect class B, A, or C beta-lactamases, respectively. Although IEF results cannot identify a specific β-lactamase, it is especially valuable for the detection of multiple β-lactamases present in an isolate. IEF can also be combined with a bioassay to detect the presence of carbapenemases by using an overlay of agar with imipenem and a second overlay with a susceptible indicator organism. Growth over an enzyme band indicates a potential carbapenemase. This is useful in detection of carbapenemases produced by *Aeromonas spp* that hydrolyze nitrocefin poorly.

3. Genotypic diction methods:

DNA hybridization: Colony blot hybridizations using labeled probes have been used to efficiently screen large numbers of clinical isolates for carbapenemase genes. Southern blot hybridization too has been used to determine whether the carbapenemase gene resides on a plasmid or the chromosome.

Polymerase chain reaction (PCR) is used to rapidly identify the carbapenemase family using specific primer pairs. Mere amplification of the genes do not reveal the complete identity of the carbapenem type, hence complete sequencing of the gene is undertaken.

Characterization of a new β-lactamase is performed by both molecular sequencing and a functional analysis of the hydrolysis and inhibition profile of the purified protein.

Prevention of spread:
International travel has resulted in rapid spread of resistant organism across the world. The prevention of spread of carbapenemase producers relies on early detection of carriers. Patients who were hospitalized while abroad and then transferred to another country should undergo screening of carbapenemase producers. Patients at risk (e.g., patients in intensive care units, transplant patients, immunocompromised patients) may also be screened. Screened patients should be kept in strict isolation before obtaining results of the screening (at least 24–48 hours). Because the reservoir of carbapenemase producers is often the intestinal flora, fecal and rectal swab specimens are collected for screening. Although commercially prepared chromomeric screening media are available, screening may also be performed on agar plates containing imipenem at a concentration of 1 µg/ml. Swabs from skin breaks or catheter sites may also be collected and inoculated in agar surface followed by placement of meropenem or ertapenem discs. Colonies forming within the zone should be examined for carbapenemase production.

High risk patients (history of previous hospitalization, travel from endemic area) must be screened on admission. Strategies must be developed for effective decontamination of equipments. In case such decontamination is not feasible, single use equipment should be used. Routine hand-hygiene must be reinforced. Equipment decontamination efficacy must be periodically assessed. All efforts should be made to minimize the spread once the infection/carriage status is known by following extra hygiene and disinfection policy. All index and secondary case contacts must also be screened and an attempt must be made to estimate the extent of spread. Awareness and training campaign for medical and nursing staff must be done. Frequent team meetings of infection control team must be held and strategies in place must be reviewed.

Treatment options against carbapenemase producers:

Aztreonam is stable to metallo-carbapenemases, including IMP, VIM and NDM. However, in isolates that also co-produce AmpC or ESBL, aztreonam is ineffective. It is also ineffective against non-metallo-beta-lactamases (OXA-48, KPC). Ceftazidime, cefotaxime and aztreonam remain active against Enterobacteriaceae with OXA-48 unless these also have AmpC or an ESBL. Temocillin is relatively stable to KPC enzymes, but MICs are mostly narrowly out of range at recommended dosage levels. Carbapenems may still be active against some producers with low-level resistance. One study demonstrated that the combination of doripenem plus ertapenem had enhanced efficacy over either agent alone. Strains with KPC, VIM, IMP and OXA-48 enzyme are variably resistant to aminoglycosides; NDM-1 producers are always resistant to all aminoglycosides. colistin, polymxyin B, tigecycline & fosfomycin have been effective in some cases.

Fosfomycin was discovered in 1968 as a secondary metabolite of several Streptomyces species. Fosfomycin, acting as a phosphoenolpyruvate analog, irreversibly inhibits phosphoenolpyruvate UDP-N-acetylglucosamine-3-O-enolpyruvyl transferase, an enzyme which catalyzes the first step of peptidoglycan biosynthesis. It can be administered both parenterally and orally.

Tigecycline, a tetracycline analogue is the first glycylcycline to be launched for clinical use. It evades the Tet(A-E) efflux pumps, which account for most acquired resistance to tetracycline and minocycline in Enterobacteriaceae and *Acinetobacter spp*.

Colistin is a multicomponent polypeptide antibiotic, comprised mainly of colistin A and B, which became available for clinical use in the 1960s. There are two forms of colistin commercially available: colistin sulfate for oral and topical use, and colistimethate sodium for parenteral use. Both can be also delivered by inhalation. Colistin sulfate is cationic, whereas colistimethate sodium is anionic. Polymyxin B differs from colistin by only one aminoacid. It is one of the last-resort antibiotics for multidrug-resistant *P. aeruginosa*, and *A. baumanni*.

In one study, a three-drug combination of aztreonam, ceftazidime and amikacin showed bacteriostatic effects against many MBL-producing strains and bactericidal effects against few strains.

Last edited on 27th May, 2012