CHAPTER – 3

REVIEW OF LITERATURE

3.1. General

Microorganisms existed on this earth millions of years before the evolution of man. Revolution in the medical sciences during the 19th century, along with the advent of antibiotics aroused a hope to do away with the microorganisms of concern to man, but today microbial resistance to antibiotics continue to be a serious threat to mankind. [Abdul Ghafur 2010].

The continuing emergence of resistant strains that cause nosocomial infections contributes substantially to morbidity and mortality in hospitalized patients. [Paramythiotou et al. 2004]. Beta lactams are the most widely prescribed antimicrobial agents in both community and hospital settings. β- lactamases cleave the β-lactam ring by hydrolysis, inactivating the antibiotic. The use of beta lactams for more than 60 years has however resulted in a dramatic increase in the rates of resistance that now threatens their value and utility. In the clinical setting, the introduction of new classes of beta-lactams has invariably been followed by the emergence of new beta-lactamases capable of degrading them, as a paradigmatic example of rapid bacterial evolution under a rapidly changing selective environment. Dissemination and increased prevalence of β-lactamases worldwide have seriously challenged their clinical effectiveness. To date, more than 890 such enzymes have been discovered [Bush et al. 2011]

The carbapenems were introduced in 1980s into clinical and they soon became the main stay of therapy for treating serious and life threatening infections caused by Enterobacteriaceae producing ESBL, Multidrug Resistant (MDR) Pseudomonas and
*Acinetobacter* species [Shanthi et al. 2009, Aloush et al. 2006, Harris et al. 1999]. But the subsequent emergence and spread of resistance to carbapenems resulted in limited therapeutic options. This resistance is mediated by the

1. Production of β-lactamases (carbapenemases) that hydrolyse the carbapenems
2. Changes in outer-membrane porins that block the entry of these antibiotics
3. Active pumping of the antibiotic out of the cell using complex “efflux pumps.”

The situation is further complicated by the fact that the “permeability” barrier and efflux mechanisms also affect other classes of antibiotics (e.g., quinolones, aminoglycosides, and tigecycline). The genes of encoding enzymes can be either chromosome- or plasmid-borne. The latter pose a threat in the context of controlling bacterial resistance, because plasmid-borne beta-lactamase genes are readily transferable among bacteria, allowing an effective and rapid spread of resistance. Recognition of the presence of a carbapenemase in a gram-negative organism is of paramount importance, since strict infection-control measures are required to avert hospital epidemics and to prevent the dissemination of these genes to other gram-negative species. [Arias, 2009]

### 3.2 Carbapenems

#### 3.2.1. Evolution of carbapenems

In the late 1960s, as bacterial β-lactamases emerged and threatened the use of penicillin, the search for β-lactamase inhibitors began in earnest. By 1976, the first β-lactamase inhibitors were discovered; these olivanic acids were natural products produced by the Gram-positive bacterium *Streptomyces clavuligerus.* This was followed by the discovery of thienamycin in 1976, produced by *Streptomyces*
The term “carbapenem” is defined as the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3 but with the substitution of carbon for sulfur at C-1. Thienamycin demonstrated potent broad-spectrum antibacterial and β-lactamase inhibitory activity. With time, enthusiasm for this compound grew rapidly, since thienamycin displayed inhibitory microbiological activity against Gram-negative bacteria, including *Pseudomonas aeruginosa*, anaerobes like *Bacteroides fragilis* and Gram-positive bacteria. These compounds were chemically unstable, so they were not used clinically.[Kattan et al. 2008, Wallace et al. 2011]

Years later, a more stable thienamycin derivative, known as imipenem, was synthesized and approved for use in 1984 [Hellinger,1999]. It became the first carbapenem approved for the treatment of complex microbial infections. Imipenem, like its parent, thienamycin, demonstrated high affinity for PBPs and stability against β-lactamases. However, imipenem was susceptible to deactivation by dehydropeptidase -1 (DHP-1), found in the human renal brush border. Therefore, co-administration with an inhibitor, cilastatin or betamipron in the ratio of 1:1 with imipenem prevented hydrolysis by DHP-1 and reduced nephrotoxicity. [Kattan et al. 2008, Wallace et al. 2011, Birnbaum J et al. 1985]. Along the journey to the discovery of more-stable carbapenems with a broader spectrum, the other currently available compounds, meropenem, biapenem, ertapenem, and doripenem were developed. A major advance in this “synthetic journey” was the addition of a methyl group to the 1-β position. Meropenem was the first carbapenem with the 1-β-methyl group and 2-thiopyrrolidinyl moiety which renders this antibiotic stable to DHP-1. [Fukasawa et al.1992]. In the subsequent decades, carbapenems with this modification were discovered, which includes biapenem, ertapenem, lenapenem and doripenem. [Bonfiglio et al. 2002]
3.2.2. Classification

Classification system for carbapenems divides them into two groups. Group 1 carbapenems, e.g. ertapenem, are defined as broad-spectrum agents that have limited/no activity against non-fermentative Gram-negative bacilli (NFGNB) and are most suited for use in treating infections caused by Enterobacteriaceae, whereas group 2 carbapenems, e.g. imipenem, meropenem and doripenem, are broad-spectrum agents that are active against NFGNB and are particularly useful in treating nosocomial infections. A third group of carbapenems has also been suggested which includes agents with activity against methicillin-resistant *Staphylococcus aureus*, such as PZ-601. [Kattan *et al.* 2008, Shah *et al.* 2003]

3.2.3. Mechanism of action

Carbapenems act by inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall. Carbapenems are not easily diffusible through the bacterial cell wall. Generally, carbapenems enter Gram-negative bacteria through outer membrane proteins (OMPs), also known as porins. After transversing the periplasmic space, carbapenems “permanently” acylate the Penicillin Binding Proteins (PBP) which catalyze the formation of peptidoglycan in the cell wall of bacteria. Carbapenems act as mechanism-based inhibitors of the peptidase domain of PBPs and can inhibit peptide cross-linking as well as other peptidase reactions. A key factor of the efficacy of carbapenems is their ability to bind to multiple different PBPs. Eventually the peptidoglycan weakens, and the cell bursts due to osmotic pressure.[Wallace *et al.* 2011, Hashizume *et al.*1984]
3.2.4. Activity of Carbapenems

Carbapenems have a broad spectrum of antimicrobial activity and are rapidly bactericidal agents because they bind with high affinity PBPs of Gram-negative bacteria. Carbapenems are the drug of choice for the treatment of infections caused by ESBL producing Enterobacteriaceae. [Rodrigues, 2011]. Carbapenems (except ertapenem) are active against clinically significant Gram-negative non-fermenters such as *P. aeruginosa*, *Burkholderia cepacia* and *Acinetobacter* spp. [Unal *et al.* 2005]. They also retain activity against streptococci, methicillin-sensitive staphylococci, *Neisseria* and *Haemophilus*. Unlike most other broad-spectrum antibiotics, carbapenems are active against most Gram-positive and Gram-negative anaerobes. Carbapenem-resistant bacteria include: ampicillin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococci*, *Stenotrophomonas maltophilia* and some isolates of *Clostridium difficile*. [Kattan *et al.* 2008]

3.2.5. Pharmacology and clinical use:

All clinically available carbapenems have low oral bioavailability and thus do not cross gastrointestinal membranes readily and must be administered intravenously; imipenem-cilastatin and ertapenem can also be delivered intramuscularly. As with other β-lactams, all of these carbapenems are eliminated predominately by renal excretion. Carbapenems are generally well tolerated. Allergic reactions to beta lactam compounds are the most common adverse events in treatment with carbapenems. [Chambers *et al.* 2005]

3.2.6. Differences among Individual Carbapenems

*Imipenem:* As the oldest of the carbapenems, imipenem is still used considerably, although it has several disadvantages as compared with newer carbapenems. It is not
approved by the US Food and Drug Administration (FDA) for meningitis, and should be avoided in the treatment of central nervous system infections because of its propensity to cause seizures in patients with elevated risk factors, e.g. renal failure or structural brain disease. It is typically very active against *P. aeruginosa* and *Acinetobacter* spp.

**Meropenem:** Meropenem has a spectrum of activity similar to that of imipenem (including *P. aeruginosa* and *Acinetobacter* spp.) and is slightly more active against Gram-negative aerobic bacteria. This agent is a substrate for the multidrug efflux system MexAB–OprM, present in *P. aeruginosa*. Overexpression of this efflux system raises the MIC of meropenem and other substrate antibiotics, but not of imipenem. The combination of a beta lactamase and down regulation of outer membrane proteins, like OprD, and an efflux system, such as Mex AB–OprM, is needed for outright resistance to meropenem to occur.

**Ertapenem:** Ertapenem possesses a longer apparent elimination half-life which allows for a convenient, once-daily administration schedule. Ertapenem is an important option for the empirical treatment of infections where a mixed flora of anaerobes and aerobes is the cause such as complicated intra-abdominal infection. It lacks antimicrobial activity against NFGNB such as *P. aeruginosa* and *Acinetobacter* spp. [Keating et al.2005].

**Doripenem:** Doripenem is a parenteral 1-betamethyl carbapenem which recommended for nosocomial pneumonia (including ventilator-associated pneumonia), complicated intra-abdominal and urinary tract infections. [Jones et al.2004].
3.3 Mechanisms of carbapenem resistance

3.3.1. Carbapenemases: Carbapenemases are specific β-lactamases with the ability to hydrolyze carbapenems. These periplasmic enzymes hydrolyze carbapenems preventing the drug from reaching the PBP target. Presently, β-lactamases are classified into four distinct classes based on structural similarities (classes A, B, C, and D) or four groups based on hydrolytic and inhibitor profiles. Class B β-lactamases use zinc to inactivate β-lactams, and all are carbapenemases. Class A, C, and D β-lactamases use a serine as a nucleophile to hydrolyze the β-lactam bond. Production of β-lactamases appears to be the most widespread cause of carbapenem resistance, since the documentation of their distribution in different bacterial species is extensive. An increasing number of class A carbapenemases (e.g., KPC and GES enzymes), class B MBLs (e.g., VIM, IMP, and NDM types), and class D carbapenemases (OXA carbapenemases) have emerged. These enzymes are encoded by mobile DNA elements with high capacity for dissemination. [Walsh 2008,2010]. MBLs are divided into three subclasses (B1, B2, and B3) on the basis of sequence similarity and structural features, with the mobile MBLs largely confined to subgroup B1. Acquired MBLs, IMP, VIM, NDM, SPM, GIM, SIM, DIM, TMB-1, FIM-1 and KHM-1 belong to the B1 subclass. AIM-1 belongs to subclass B3. [Leiros 2012, Bush 2010].

In addition, overproduction of class C β-lactamases (Amp C betalactamases), can lead to carbapenem resistance, especially when combined with other resistance mechanisms (e.g., porin loss). [Rodriguez-Martinez et al.2009,Mammeri et al.2010]

3.3.2. Efflux pumps:

Active efflux of toxins and chemicals out of the bacterial cell is a mechanism that protects them from the adverse effects of their environment. Antibiotics used in clinical settings are among those toxic compounds and extrusion of antibiotics from
bacterial cells significantly decreases their clinical utility. Antibiotics are expelled from the cells by the membrane transporter proteins, the efflux pumps. Most of the genes encoding these multidrug resistant pumps are normal constituents of the bacterial chromosomes. In some of these genes have high constitutive expression and confer intrinsic resistance to the antibiotics while in others the genes are expressed after acquisition of regulatory mutations. [Lomovskaya et al. 2001]. Efflux pumps are grouped into several superfamilies: the small multidrug resistance (SMR) superfamily, the resistance-nodulation-division (RND) superfamily, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamily, and the multidrug and toxic compound extrusion (MATE) superfamily. Carbapenem resistance due to overexpression of efflux porins, is reported mostly for \textit{P. aeruginosa}, \textit{Enterobacter aerogenes} and \textit{A. baumannii}. [Wallace et al. 2011]. Due to its hydrophobic nature, meropenem is a substrate for the Mex AB–Opr M system comprising a pump (Mex B), a linker lipoprotein (Mex A) and an exit portal (Opr M). [Quale et al. 2006]. Over expression of the Mex AB-Opr M efflux system affects efficacy or meropenem but not that of imipenem. In addition the Mex CD- opr J and Mex XY-Opr M efflux systems are involved in reduced susceptibility to meropenem [Pai et al.2001, Rodriguez-Martinez et al.2009]

3.3.3. Loss of porins

In porin loss, the loss of a membrane protein channel decreases the rate of entry of antibiotics into the periplasm, thus raising the MIC. Substitutions in, or decreased expression of, porins resulting in decreased entry of carbapenems into the periplasm exists in \textit{P. aeruginosa}, \textit{K. pneumoniae}, \textit{Enterobacter aerogenes}, \textit{E. coli}, \textit{Serratia marcescens}, \textit{Proteus mirabilis}, \textit{Citrobacter freundii}, \textit{A. baumannii}, \textit{Enterobacter cloacae}, \textit{Proteus rettgeri}, \textit{Shigella dysenteriae} and \textit{Salmonella enterica}.  

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Loss of Opr D confers resistance to imipenem and low grade resistance to meropenem [Pai et al. 2001]. If combined with beta lactamase production, porin loss confer resistance to one or many antibiotics simultaneously. An example of this mechanism is the loss of a specific porin known as Opr D in *P. aeruginosa* with simultaneous production of Amp C confers resistance to imipenem [Livermore 2002].

### 3.3.4. Penicillin binding proteins (PBP)

Mutations in the PBP protein and/or decreases in PBP transcription also result in carbapenem-resistant phenotypes in *P. aeruginosa*, *A. baumannii* and *Proteus mirabilis*. In addition, amino acid substitutions in PBPs or acquisition of a novel PBP can cause carbapenem resistance in *Proteus mirabilis*, *E. coli* and *P. aeruginosa*. [Wallace et al. 2011]

### 3.4. Carbapenem resistance in Enterobacteriaceae

Enterobacteriaceae are rod-shaped, Gram-negative bacilli that are normal inhabitants of the intestinal flora and among the most common human pathogens, causing infections that range from cystitis to pyelonephritis, septicaemia, pneumonia, peritonitis, meningitis, and device-associated infections. They are the most common cause of both community and hospital acquired infections, with *Escherichia coli* being by far the most important pathogen for humans. Enterobacteriaceae spread easily between humans by hand carriage as well as contaminated food and water and have a propensity to acquire genetic material through horizontal gene transfer, mediated mostly by plasmids and transposons. This combination is why emerging multidrug resistance in Enterobacteriaceae is of the utmost importance for clinical therapy [Nordmann et al. 2012b, 2012a]. Among the Enterobacteriaceae, *Klebsiella*
*pneumoniae* is an important cause of nosocomial infections and a notorious collector of multidrug resistant plasmids. It persists and spreads rapidly in the health care settings resulting in outbreaks, due to its efficiency to colonise and enhanced ability to acquire resistance to antibiotics. During the ESBL era, *K. pneumoniae* was the index species for plasmids encoding ESBLs and a variety of genes encoding resistance to aminoglycosides and fluoroquinolones. [Tzouvelekis *et al.*2012]. The other common Enterobacteriaceae causing infections in humans include *Citrobacter* species, *Enterobacter* species, *Serratia marcescens*, *Proteus* spp and *Providencia* spp. They cause a wide variety of infection such as septicemia, skin and soft tissue, urinary tract and respiratory tract infections [Chen *et al.* 2011a, Yang *et al.* 2011]. Since 2000, the spread of Enterobacteriaceae isolates producing ESBLs capable of hydrolysing almost all beta lactams except carbapenems has been reported worldwide. The consequence of this phenomenon has been an increased consumption of carbapenems. [Nordmann P *et al.* 2011a]

In Enterobacteriaceae, carbapenem resistance arises from two main mechanisms

- Acquisition of carbapenemase genes that encode for enzymes capable of degrading carbapenems
- Decrease in the uptake of antibiotics by a qualitative and/or quantitative deficiency of porin expression in association with overexpression of beta lactamases that possess very weak affinity for carbapenems

The most important carbapenemases in Enterobacteriaceae are categorized as three types of enzymes:

i. KPC type enzymes (Ambler Class A)
ii. VIM, IMP, and NDM metallo beta lactamases (Ambler Class B)
iii. OXA-48 type enzyme and its variants (Ambler Class D)

3.4.1. Non Carbapenemase mediated carbapenem resistance in Enterobacteriaceae

The primary porins in Enterobacteriaceae involved in taking up antibiotics belong to the Omp F or Omp C families. Thus, any changes in the number or activity of bacterial porins might have an effect on antibiotic resistance. Mutation of a gatekeeping loop or central channel in the porin proteins, a loss of porin expression, or a shift in the types of porins found in the outer membrane can decrease sensitivity to antibiotics. Porin synthesis may be regulated in response to antimicrobial compounds or aromatic products via several cascades involving the mar and sox operons, with a subsequent decrease in the number of porins in the outer membrane. [Pages et al. 2008, Nikaido 2003]

Carbapenem resistance was first observed in Enterobacter spp. (approximately 1% of the strains) overexpressing a chromosomal amp C gene encoding an intrinsic cephalosporinase and exhibiting modifications in their Omp C or Omp F porins [Doumith et al. 2009, Lavigne et al. 2012; Martinez-Martinez et al. 2008; Tzouuvelekis et al. 1994]. Similar mechanisms were reported for Serratia sp., Citrobacter freundii and Morganella morganii. This has been observed in other Enterobacteriaceae species that do not express an intrinsic cephalosporinase, such as E coli, K. pneumoniae and Salmonella spp. In those cases, resistance corresponds to a combination of plasmid-encoded Amp C expression together with decreased cell membrane permeability owing to modifications in Omp K35/36 for K. pneumoniae, and in Omp F and Omp C for E. coli. [Chia et al. 2009, Shin et al. 2011].
Similarly, production of an ESBL in combination with outer membrane permeability defects can be responsible for carbapenem resistance in Enterobacteriaceae [Doumith et al. 2009, Lartigue et al. 2007, Garcia-Fernanadez et al. 2010]. Strains that do not produce carbapenemase but which are carbapenem resistant are usually less resistant to antibiotics of other families. Their carbapenem resistance trait is not transferable, as opposed to most of the strains harboring carbapenemase genes. For this reason, carbapenem-resistant isolates that do not produce carbapenemases are considered of less clinical concern than carbapenemase-producing strains.[Nordmann P et al. 2012 b]

3.4.2. Carbapenemase-mediated carbapenem resistance in Enterobacteriaceae

The first carbapenemases identified in Enterobacteriaceae were SME-1 in London in 1982 and IMI-1 in the USA in 1984. The first carbapenemase reported as a serine carbapenemase in the primary literature was the chromosomally encoded Nmc A from an Enterobacter cloacae clinical isolate. A large variety of carbapenemases belonging to three molecular classes of β-lactamases have been identified in Enterobacteriaceae: the Ambler class A, class B and class D β-lactamases. In addition, rare chromosome-encoded cephalosporinases (Ambler class C or Amp C) produced by Enterobacteriaceae may possess slight extended activity toward carbapenems, but their clinical significance remains uncertain. [Nordmann et al. 2011a, 2012 b]

3.4.2.1. Class A Carbapenemases in Enterobacteriaceae

Class A serine carbapenemases of functional group 2f have appeared sporadically in clinical isolates since their first discovery over 20 years ago. These β-lactamases have been detected in Enterobacter cloacae, Serratia marcescens, and Klebsiella spp. as single isolates or in small outbreaks. Bacteria expressing these
enzymes are characterized by reduced susceptibility to imipenem, but Minimum inhibitory concentration (MIC) can range from mildly elevated (e.g., imipenem MICs of $\leq 4$ µg/ml) to fully resistant. These $\beta$-lactamases, therefore, may go unrecognized following routine susceptibility testing. [Queenan et al.2007, Walther Rasmussen et al.2007]

Three major families of class A serine carbapenemases include the NMC/IMI, SME, and KPC enzymes. Their hydrolytic mechanism requires an active-site serine. All have the ability to hydrolyze a broad variety of $\beta$-lactams, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanate and tazobactam, placing them in the group 2f functional subgroup of $\beta$-lactamases. The GES type beta lactamases was originally considered a classical ESBL family because GES-1 does not possess carbapenemase activity, but variants have subsequently been identified that possess weak but significant carbapenemase activity. [Queenan et al.2007, Walther Rasmussen et al.2007, Nordmann et al.2012b]

SME (Serratia marcescens enzyme): The SME enzymes have been only identified in Serratia marcescens. This family includes three variants (SME-1, -2, and -3), all chromosomally-encoded, that are recovered sporadically throughout the USA. [Nordmann et al., 2012b]

IMI and NMC-A: The IMI (for “imipenem-hydrolyzing $\beta$-lactamase”) and NMC-A (for “not metalloenzyme carbapenemase”) enzymes have been detected in rare clinical isolates of E. cloacae in the United States, France, and Argentina. These enzymes are chromosomally encoded and can be induced. [Walther Rasmussen et al. 2007]

GES: The GES (Guiana Extended spectrum) enzyme was first reported from Greece in a K. pneumoniae isolate. The genes encoding are located in plasmids and are
mainly found in *P. aeruginosa* and also in members of Enterobacteriaceae family. The GES family now includes 20 variants. Some GES variants possess amino acid substitutions within their active sites that enlarge their spectrum of activity against carbapenems. GES-4, -5, and -6 have been reported in Enterobacteriaceae, whereas GES-2, -11, and -14 are restricted to Pseudomonads or *Acinetobacter*. Although rare, GES enzymes have been identified worldwide, with reports from Greece, France, Portugal, South Africa, French Guiana, Brazil, Argentina, Korea, and Japan. [Walther Rasmussen J *et al.* 2007]

**KPC:** The KPC enzymes are named so as they were first detected in *K. pneumoniae*. They hydrolyse beta lactams of all classes. Of the functional group 2f carbapenemases, the KPC family has the greatest potential for spread as it is located in the plasmids of *K. pneumoniae*, an organism notorious for its ability to accumulate and transfer resistance determinants. KPC-1 is also found in other Enterobacteriaceae and *P. aeruginosa*. Outbreaks due to KPC 2 producing *K. pneumoniae* have been reported from many parts of United States. KPC enzymes are currently the most clinically significant enzymes among class A betalactamases. The first KPC producing strain (KPC-2 in *K. pneumoniae*) was identified in 1996 in the Eastern part of the USA. Since then, 11 KPC variants have been identified (KPC-2 to KPC-12). Within a few years, KPC-producing strains disseminated widely and have been identified in United States as well as Puerto Rico, Columbia, Italy, Greece, Israel, and China. Outbreaks of KPC-producing strains have also been reported in many European countries and in South America. [Navon Venezia *et al.* 2009, Nordmann *et al.* 2009]. Although community-acquired KPC-producing strains have been reported in the USA and Israel a few years ago they remain rare. Indeed, KPC enzymes have been reported mostly from nosocomial *K. pneumoniae* isolates and to a much lesser
extent from other Enterobacteriaceae species. [Nordmann et al. 2009, Borer et al. 2009, Patel et al. 2009, Schwaber et al. 2008]. There is striking evidence that the bla KPC genes are always associated with the genetic element, transposon Tn4401. [Cuzon et al. 2010]

3.4.2.2. MBL in Enterobacteriaceae

Class B betalactamases exhibit a broad spectrum of hydrolytic activity including all penicillins, cephalosporins, and carbapenems, with the exception of monobactam, aztreonam. Their activity is not inhibited by beta lactamase inhibitors such as clavulanic acid, tazobactam, or sulbactam. MBLs are betalactamases that require divalent cations such as zinc as metal cofactors for enzyme activity. They are inhibited by chelators such as Ethylene diamine tetracetic acid (EDTA).

The first MBLs were detected from environmental and opportunistic bacteria such as Bacillus cereus, Aeromonas spp. and Stenotrophomonas maltophilia. Those bacteria are mostly opportunistic and soil dwelling pathogens and their MBL genes are intrinsic and chromosome-borne. Since the 1990s, a dramatic increase in acquired or transferable MBL genes has been described in Pseudomonas spp. and Enterobacteriaceae. [Walsh et al. 2005, Bush 1998]

Class B MBLs are mostly of the Verona integron–encoded metallo-β-lactamase (VIM) and IMP (Imipenemase) types and, more recently, of the New Delhi metallobetalactamase-1 (NDM-1) type. The other acquired MBLs include SPM-1 (Sao Paulo metallo-β-lactamase), GIM-1 (German Imipenemase), SIM-1 (Seoul Imipenemase), DIM-1 (Dutch Imipenemase) and AIM-1 (Adelaide Imipenemase). [Nordmann et al. 2012 b, Cornaglia 2011]
The first acquired MBL, IMP-1, was reported in Serratia marcescens in Japan in 1991. Since then, MBLs have been described worldwide. Endemicity of VIM- and IMP-type enzymes has been reported in Greece, Taiwan, and Japan although outbreaks and single reports of VIM and IMP producers have been reported in many other countries. Most MBL producers are hospital acquired and multidrug-resistant K. pneumoniae. [Walsh et al. 2005, Queenan et al. 2007, Bush 1998]. Resistance levels to carbapenems of MBL producers may vary. Death rates associated with MBL producers range from 18% to 67% [Nordmann et al.2011a, 2012 b, Daikos et al.2009].

**IMP**: IMP type beta lactamases were among the first acquired MBLs identified and were detected in *Pseudomonas* spp., *Acinetobacter* spp., and Enterobacteriaceae [Zhao et al. 2011b]. In Enterobacteriaceae, IMP-1 was first reported from a *S. marcescens* isolate in Japan in 1991. [Walsh et al. 2005]. Since then, 33 IMP variants have been assigned and IMP type carbapenemase producers have spread worldwide. The endemic spread of IMP type enzymes has been reported from Japan, Taiwan, East of China, and more recently from Greece, although outbreaks and single reports have been reported in many other countries. It has been suggested that selection of IMP type genes occurred first in Japan as a consequence of heavy carbapenem usage. Analysis of the *bla* _IMP_ genetic environments most often revealed features of class 1 integrons which are DNA-based structures that harbor antibiotic resistance genes, named gene cassettes, and are co-expressed from a single promoter. Resistance genes encode decreased susceptibility to unrelated antibiotic molecules (e.g. other betalactams, aminoglycosides, sulfonamides, and chloramphenicol). They are usually identified inside transposon structures, thus allowing their spread. [Zhao et al. 2011b, Nordmann et al.2012b, 2011a]
**VIM:** Another family of MBLs includes the VIM enzymes. VIM-1 was first identified in Italy in 1997, and shortly after the VIM-2 variant was reported in France from a *P. aeruginosa* isolate [Walsh et al. 2005]. The VIM group of enzymes contains 33 variants, mainly identified from *P. aeruginosa* and Enterobacteriaceae isolates such as *K. pneumoniae*, *Citrobacter freundii*, *Enterobacter cloacae* and *Serratia marcescens*. The genes also correspond to gene cassettes located inside class 1 integrons. VIM-2 is the most common MBL type reported worldwide, with an endemic spread in Southern Europe and Southeast Asia. [Zhao et al. 2011a]

**Other MBLs:** The other acquired MBLs include SPM-1, GIM-1, SIM-1, DIM-1, and AIM-1; however, these enzymes have so far not been identified in Enterobacteriaceae strains. [Nordmann et al. 2012b]. Recently, the beta lactamase KHM-1 was identified in Japan from a single *Citrobacter freundii* clinical isolate.[Sekiguchi et al. 2008]

**NDM-1:** NDM-1 is the most recently discovered transferable molecular class B beta lactamase. Discovered in 2008 in Sweden from an Indian patient previously hospitalized in New Delhi, India, NDM-1- producing Enterobacteriaceae are now the focus of worldwide attention.[Yong et al. 2009, Struelens et al. 2010, Johnson et al. 2013, Nordmann et al. 2012b] NDM-1 has been reported from many countries and its origin has been traced to Asia.[Deshpande et al. 2010, Kumarasamy et al. 2010, Nordmann et al. 2011b, 2011c]. It has been claimed that most of the patients infected or colonized by NDM-1 producers had traveled to the Indian subcontinent or have some links with India, Pakistan, or Bangladesh. These data indicate that the Indian subcontinent is a reservoir of *blaNDM-1* genes. However, there are only sparse reports of single center molecular epidemiological studies done on NDM-1 producers particularly from the Indian subcontinent. [Nagaraj et al. 2012, Castanheira et al. 2011a, Lascols et al. 2011]. Interestingly several NDM-1 producing
Enterobacteriaceae isolates have been reported in patients having a link to the Balkan states or the Middle East, suggesting that those areas also might constitute another reservoir of NDM-1-producing strains [Livermore et al. 2011, Poirel et al. 2011a]. The \textit{bla}\textsuperscript{NDM-1} gene is not associated with a single clone, a single species, or to a specific plasmid backbone but has been identified from unrelated Gram negatives, mostly Enterobacteriaceae species, harbored by different plasmid types [Poirel et al. 2011b]. Plasmids carrying the \textit{bla}\textsuperscript{NDM-1} gene are diverse in size, incompatibility group, and associated resistance genes. Noticeably, NDM-1-producing strains may express many other unrelated resistance genes, such as those that encode other carbapenemases (OXA-48 type, VIM type), Amp C cephalosporinas, ESBLs, and resistance to aminoglycosides (16sRNA methylases), macrolides (esterases), rifampicin (rifampicin- modifying enzymes), and sulfamethoxazole. The association of such a high number of plasmid- and chromosome encoded resistance genes (including resistance to fluoroquinolones) in single isolates had been observed and explains the multidrug resistance pattern of the NDM-1 producers. Many NDM-1 producers remain susceptible only to tigecycline, colistin, and to a lesser extent to fosfomycin [Nordmann et al. 2011a, 2012b].

Although \textit{bla}\textsuperscript{NDM-1}-positive isolate are predominantly nosocomial \textit{K. pneumoniae} isolates, it has been identified in community-acquired \textit{E. coli} [Nordmann et al. 2012b]. NDM-1 producing Enterobacteriaceae are implicated in a wide range of clinical conditions such as bacteremia, peritonitis, ventilator associated pneumonia, device associated infections, soft tissue and urinary tract infections. Both hospital and community acquired infections have been reported. Colonization of the colon precedes infection and faeco-oral transmission is the route of transmission. NDM-1 has been reported in Enterobacteriaceae other than \textit{E. coli} and \textit{K. pneumoniae}, which
include *Klebsiella oxytoca, Proteus mirabilis, Morganella morganii, Enterobacter cloacae, Citrobacter freundii* and *Providencia* spp.

In Indian studies, the prevalence of NDM-1 producers among carbapenem resistant Enterobacteriaceae ranged between 31.2% and 91.6%. [Deshpande *et al.* 2010, Kumarasamy *et al.* 2010, Nagaraj *et al.* 2012, Castanheira *et al.* 2011a, Lascols *et al.* 2011]. Most NDM-1 producers remain susceptible to tigecycline and polymyxins. However, tigecycline may not reach desired serum levels to treat systemic infection leaving polymyxins as the last resort [Nordmann *et al.* 2011b]

Currently, 7 NDM variants have been identified. Although most NDM-producing strains described are Enterobacteriaceae species, some NDM-2 variant has been identified in *A. baumannii*. [Nordmann *et al.* 2011b, Johnson *et al.* 2013]

A recent study focused on the genetic environment of *bla* NDM-1 revealed a strong association between the genes *bla*NDM-1 and *ble*MBL, the latter gene encoding a functional bleomycin resistance protein. [Nordmann *et al.* 2012b, Dortet *et al.* 2012a].

### 3.4.2.3. OXA carbapenamases in Enterobacteriaceae

Class D beta-lactamases, also namedOXAs for ‘oxacillinases’, include 232 enzymes, with a few variants possessing some carbapenemase activity. Although most of the OXA carbapenemase variants have been identified in *Acinetobacter* spp., OXA-48 has been found only in Enterobacteriaceae. [Nordmann *et al.* 2012b]

The class D, carbapenem hydrolyzing OXA-48 was first reported from Turkey in *K. pneumoniae* [Carrer *et al.* 2008]. Since then, outbreaks linked to OXA-48 have been described worldwide. Variants of OXA-48 that differ by substitution of one or more amino acids were identified subsequently namely, OXA-163, OXA-181, OXA-204 and OXA-232. Of these, so far only OXA-181 variant possess significant carbapenemases activity and has been found to be associated with other
carbapenamase encoding genes such as \( \text{bla}_{\text{NDM-1}} \) and \( \text{bla}_{\text{VIM}} \). OXA-181, displays 45 nucleotide substitutions leading to 4 amino acid differences compared to OXA-48.[Poirel et al. 2012a]. World over OXA-48 and its variant OXA-181 have been reported in several species of Enterobacteriaceae, particularly in \( K. \text{pneumoniae} \). Case reports referring to infections caused by OXA-181 producers have been described from The Netherlands, Singapore, France and Sultanate of Oman.[Potron et al.2011, Koh et al.2012, Poirel et al.2011c]. The acquisition of the causative strains have all been traced to the Indian subcontinent.In the SENTRY study conducted between 2006-07, 25.64% of the carbapenem resistant Enterobacteriaceae were found to harbor OXA-181.[Castanheira et al. 2011a]. In contrast, data from the SMART study 2009 on the Indian isolates indicate that OXA-48 is present only in about 2% of the carbapenem resistant Enterobacteriaceae causing intra-abdominal infections [Lascols et al. 2011].

The OXA-48 carbapenamase and its variants hydrolyze penicillins at a high level, but hydrolyze carbapenems at low level only and show very weak activity against extended spectrum cephalosporins. Hence the organisms producing \( \text{bla}_{\text{OXA-48}} \) exhibit reduced susceptibility to carbapenems and their MIC to carbapenems in-vitro may remain in the susceptible range thus making their detection problematic. In such infections, treatment with carbapenems, result in adverse outcomes. Their detection is therefore crucial to institute appropriate therapy and initiate preventive measures.[Paño-Pardo et al.2013, Poirel et al.2012a]. The suspicion of presence of \( \text{bla}_{\text{OXA-48}} \) is often very complicated because some of them may be carbapenem resistant but remain susceptible to cephalosporins subclass III which may be regarded as the potential treatment of choice. But this is a rare phenomenon in countries where ESBL is a major resistance mechanism in Enterobacteriaceae conferring resistance to
the cephalosporins subclass III. Thus the resistance pattern becomes broader leaving only limited therapeutic options such as polymyxins and tigecycline. [Dimou et al. 2012]

3.5. Carbapenem resistance in NFGNB:

NFGNB are a group of aerobic, non-spore forming Gram negative bacilli that either do not utilize carbohydrates as a source of energy or degrade them through oxidative pathways. The NFGNB includes taxonomically diverse organisms and are important nosocomial pathogens capable of causing invasive infections in critically ill and immunocompromised patients, complicate treatment, adversely affect clinical outcomes and patient treatment costs. [Koneman et al. 2006].

*Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the most common NFGNB pathogenic for humans and are of greatest concern for hospitalized patients particularly those in ICU, where these pathogens are capable of causing severe invasive infections in critically ill and immunocompromised patients. [Karlowsky et al. 2003]. Their ability to survive under a wide range of environmental conditions make them a frequent cause of outbreaks of infection and endemic health care associated pathogens.[Maragakis et al.2008, Peleg et al.2008, Lister et al.2009]. Infections caused by other species of NFGNB are relatively infrequent. Some of the infrequently isolated NFGNB are *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, and *Chryseobacterium meningosepticum*. [Taneja et al.2003].

Multidrug resistant *P. aeruginosa* and *A. baumannii* cause a multitude of infections that include bacteraemia, pneumonia, meningitis, skin and soft tissue and urinary tract infections. Selection of the most appropriate antibiotic is complicated by their ability to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection. Carbapenems are the drugs of choice in the
treatment of infections caused by multidrug resistant *P. aeruginosa* and *A. baumannii*. Emergence and spread of carbapenem resistance limits therapeutic options to polymyxins and tigecycline.[Meletis et al. 2012, Peleg et al. 2008]

Epidemiological outcome studies have shown that infections caused by carbapenem resistant *P. aeruginosa* and *A. baumannii* are associated with significant increases in morbidity, mortality, need for surgical intervention, length of hospital stay and chronic care, and overall cost of treating the infection.[Maragakis et al. 2008, Peleg et al. 2008, Lister et al. 2009].

Resistance to carbapenems is mediated by lack of drug penetration (i.e., porin mutations and efflux pumps) and/or carbapenem hydrolysing beta lactamase enzymes (carbapenemases)

### 3.5.1. Carbapenem resistance in *Pseudomonas aeruginosa*

1. Production of carbapenem hydrolyzing enzymes (carbapenemases)
2. Increased production of Amp C chromosome encoded cephalosporinas
3. Reduced outer membrane porin Opr D expression
4. Overexpression of efflux pumps

#### 3.5.1.1. Carbapenemases in *Pseudomonas aeruginosa*

Carbapenemases mediating carbapenem resistance in *Pseudomonas aeruginosa* belong to Ambler Class A and Class B

**Class A carbapenemases in *P. aeruginosa***: The first report of KPC-producing *P. aeruginosa* isolates was described in three genetically related isolates from Colombia in 2007. The spread of *bla* _KPC_ into different genera is most likely associated with its residence within mobile genetic elements on plasmids of various sizes. The genes encoding the KPC carbapenemases all are plasmid-borne, apart from the *bla* _KPC_ in

The GES/IBC family of β-lactamases is an infrequently encountered family that was first described in 2000 with reports of IBC-1 from an *E. cloacae* isolate in Greece and GES-1 in a *K. pneumoniae* isolate from French Guiana. [Giakkoupi *P et al.* 2000, Poirel *et al.* 2000a]. The genes encoding the GES family of enzymes were located in integrons on plasmids. Because the enzymes had a broad hydrolysis spectrum that included penicillins and extended-spectrum cephalosporins, they were initially classified as ESBLs. Their hydrolysis spectrum was expanded in 2001 to include imipenem, with the report of GES-2 in a clinical isolate of *P. aeruginosa*. [Poirel *et al.* 2001a, Queenan *et al.* 2007]. At least nine GES variants have been described, with GES-9 recently identified in a *P. aeruginosa* isolate from France. [Poirel *et al.* 2005]. Although rare, GES enzymes have been identified worldwide, with reports from Greece, France, Portugal, South Africa, French Guiana, Brazil, Argentina, Korea, and Japan. These enzymes have been most frequently associated with single occurrences. However, *P. aeruginosa* strains expressing GES-2 have caused a small nosocomial outbreak in eight patients. [Queenan *et al.* 2007, Castanheira *et al.* 2004b, Pasteran *et al.* 2005, Poirel *et al.* 2002]

**MBLs in *P. aeruginosa***: In general, carbapenem resistance in *P. aeruginosa* attributed to β-lactamases is due to MBL. Production of MBL by *P. aeruginosa* leads to resistance to all beta lactams except the monobactams such as aztreonam. In contrast to the chromosomal MBL, whose presence is directly correlated with the prevalence of the producing species, there has been a dramatic increase in the detection and spread of the acquired or transferable families of these metalloenzymes. The most common MBL families include the VIM, IMP, SPM, GIM and SIM
enzymes, which are located within a variety of integron structures, where they have been incorporated as gene cassettes. When these integrons become associated with plasmids or transposons, transfer between bacteria is readily facilitated [Queenan et al. 2007, Strateva et al. 2009, Bonomo et al. 2006]. Since their initial discoveries, SPM, GIM, and SIM metallo-β-lactamases have not spread beyond their countries of origin. However, VIM and IMP continue to be detected worldwide, with an overall trend of these two MBLs moving beyond \( P. aeruginosa \) and into the Enterobacteriaceae [Queenan et al. 2007]. In Asia, \( bla_{\text{IMP}} \) and \( bla_{\text{VIM}} \) are prevalent. \( Bla_{\text{IMP}} \) is found mainly in Japan, Korea, China, Taiwan, and Iran [Fang et al. 2008, Peymani et al. 2011, Franco et al. 2010]. The prevalence of MBL in India has ranged from 7% to 65% among carbapenem-resistant \( P. aeruginosa \) and \( bla_{\text{VIM}} \) type was the most common [Manoharan et al. 2010, Toleman et al. 2007, Castanheira et al. 2009].

The different MBL types found in \( P. aeruginosa \) is shown in table -1

**IMP:** The first carbapenemase proven in \( P. aeruginosa \) was IMP-1. It was found in Japan in a large-scale study of carbapenem-resistant clinical isolates during 1992–1994 [Senda et al. 1996]. The gene was localized to a large plasmid (36 kb) and found to be part of a gene cassette within a class 1 integron. [Strateva et al. 2009]. From 2000 until 2001 other IMP variants of MBLs were found in various Gram-negative bacteria worldwide. \( Bla_{\text{IMP-7}} \) were identified among \( P. aeruginosa \) clinical isolates in Canada [Parkins et al. 2007] and Singapore (Koh et al. 2004), and \( bla_{\text{IMP-9}} \) was found in China [Xiong et al. 2006], and \( bl{a}_{\text{IMP-13}} \) in Italy (Pagani et al. 2005). In 2002, \( bl{a}_{\text{IMP-16}} \) MBL was found in a \( P. aeruginosa \) strain from Brazil [Mendes et al. 2004]. Currently, the most recent IMP MBL (IMP-18) was found in a \( P. aeruginosa \) clinical isolate in the USA [Hanson et al. 2006].
**VIM**- VIM-1 carbapenemase, found in a nosocomial *P. aeruginosa* strain isolated at the Verona University Hospital, Italy, in 1997, is the first representative of a new family of acquired MBLs [Lauretti *et al.* 1999]. Although VIM-1 shows less than 30% amino acid identity to IMP enzymes, it has the same extended spectrum of hydrolysis [Nordmann 2002]. Like *bla* IMP genes, *bla* VIM-1 is a part of a gene cassette inserted in the In70 class 1 integron, which carries multiple resistance encoding genes following genes.[Riccio *et al.*2001]. In 2004–2005 Corvec *et al.* (2006) detected *P. aeruginosa* clinical isolates producing VIM-1 from different French hospitals.

VIM-2 was originally identified in a *P. aeruginosa* blood stream isolate from a patient with neutropenia in France.It was closely related to VIM-1 MBL reported from Italian *P. aeruginosa* clinical isolates. [Poirel *et al.* 2000b]. *In P. aeruginosa*, VIM-2 is now the most widespread MBL reported from several countries [Strateva *et al.*2009]. The *bla* VIM-2 allele was found to be carried on mobile elements known as gene cassettes and they are inserted into class 1 integrons [Poirel *et al.*2000 b, 2001b; Yu *et al.* 2006]. Integron-located resistance genes provide them with an increased potential for expression and dissemination. VIM-3 metalloenzyme was identified in a *P. aeruginosa* isolate in Taiwan (Yan *et al.* 2001). The following discoveries of VIM-type MBLs in *P. aeruginosa* isolates were made: VIM-4 in Greece, Hungary, Poland and Sweden ; VIM-5 in Turkey ; VIM-7 in the USA ; VIM-8 in Columbia ; VIM-11 in Argentina and Italy ; VIM-13 in Spain ;VIM-15 in Bulgaria and VIM-16 in Germany.[Strateva *et al* 2009].

**SPM** : SPM-1 was first isolated in a *P. aeruginosa* strain in Sao Paolo, Brazil. Since the initial report, single clones of SPM-1-containing *P. aeruginosa* have caused

**GIM:** GIM-1 was isolated in Germany in 2002. GIM had approximately 30% homology to VIM, 43% homology to IMPs, and 29% homology to SPM. GIM-1 has characteristics similar to those of the other acquired MBL in that it was found in five clonal *P. aeruginosa* isolates within a class 1 integron on a plasmid.[Castanheira et al. 2004a, Strateva et al. 2009]

**NDM-1:** At the Military Medical Academy in Serbia, routine analysis of carbapenemase producing bacterial isolates, revealed NDM-1 in seven clinical isolates of *P. aeruginosa*. The source patients were hospitalized in Serbia and had no history of travel to any other country. Subsequently, in 2012 France reported recurrent pyelonephritis due to NDM-1 producing *P. aeruginosa*. This patient had history of prior hospitalization in Serbia and gave rise to the hypothesis that the Balkan states may be endemic for NDM-1 producers [Jovicic et al. 2011, Nordmann et al. 2011b, Flateau et al. 2012].

**AIM-1:** Three clinical *P. aeruginosa* isolates, from Adelaide, Australia, exhibited a positive MBL screen test but PCR negative for known MBL genes. A gene bank was created, and an MBL gene, designated *bla* AIM-1, was cloned and fully characterized.[Leiros 2012, Yong D et al. 2012]
FIM-1: A novel acquired MBL, named FIM-1, was identified and characterized from a multidrug-resistant *P. aeruginosa* clinical isolate cultured from a patient with a vascular graft infection in Florence, Italy. [Pollini *et al* 2013].

### Table-1: MBLs found in *P. aeruginosa*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Geographical dissemination</th>
<th>Location of encoding gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IMP-type</strong></td>
<td></td>
<td>Integrons in plasmid or chromosome</td>
</tr>
<tr>
<td>IMP-1</td>
<td>Japan</td>
<td></td>
</tr>
<tr>
<td>IMP-7</td>
<td>Singapore</td>
<td></td>
</tr>
<tr>
<td>IMP-9</td>
<td>Canada, Singapore</td>
<td></td>
</tr>
<tr>
<td>IMP-13</td>
<td>China</td>
<td></td>
</tr>
<tr>
<td>IMP-16</td>
<td>Italy</td>
<td></td>
</tr>
<tr>
<td>IMP-18</td>
<td>Brazil, USA</td>
<td></td>
</tr>
<tr>
<td><strong>VIM-type</strong></td>
<td></td>
<td>Integrons in plasmid or chromosome</td>
</tr>
<tr>
<td>VIM-1</td>
<td>Italy, France, Greece</td>
<td></td>
</tr>
<tr>
<td>VIM-2</td>
<td>France, Italy, Greece, Spain, Germany, Portugal, Poland, Russia, Ireland, Turkey, Venezuela, Korea, Japan, China, Saudi Arabia, India, USA, Columbia, Canada</td>
<td></td>
</tr>
<tr>
<td>VIM-3</td>
<td>Taiwan</td>
<td></td>
</tr>
<tr>
<td>VIM-4</td>
<td>Greece, Hungary, Poland, Sweden</td>
<td></td>
</tr>
<tr>
<td>VIM-5</td>
<td>Turkey</td>
<td></td>
</tr>
<tr>
<td>VIM-7</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>VIM-8</td>
<td>Columbia</td>
<td></td>
</tr>
<tr>
<td>VIM-11</td>
<td>Argentina, Italy</td>
<td></td>
</tr>
<tr>
<td>VIM-13</td>
<td>Spain</td>
<td></td>
</tr>
<tr>
<td>VIM-15</td>
<td>Bulgaria</td>
<td></td>
</tr>
<tr>
<td>VIM-16</td>
<td>Germany</td>
<td></td>
</tr>
<tr>
<td><strong>Other MBLs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM-1</td>
<td>Serbia</td>
<td>Plasmid and integron borne</td>
</tr>
<tr>
<td>SPM-1</td>
<td>Brazil</td>
<td>Plasmid borne</td>
</tr>
<tr>
<td>GIM-1</td>
<td>Germany</td>
<td>Plasmid and integron borne</td>
</tr>
<tr>
<td>AIM-1</td>
<td>Australia</td>
<td>Plasmid and integron borne</td>
</tr>
<tr>
<td>FIM-1</td>
<td>Italy</td>
<td>Plasmid and integron borne</td>
</tr>
</tbody>
</table>
3.5.1.2. Efflux mediated carbapenem resistance in *P. aeruginosa*

Active efflux is an important non-enzymic mechanism of β-lactam resistance in *P. aeruginosa*. Efflux also contributes to the development of multiple resistances to all antipseudomonal antibiotics and is mediated by four genetically different three-component efflux systems that belong to the resistance–nodulation–division (RND) family: MexA–MexB–OprM, MexC–MexD–OprJ, MexE–MexF–OprN and MexX–MexY–OprM. (table-2) [Strateva T *et al.* 2009, Livermore 2001, 2002].

**Table-2: Structure and substrate specificity of the three-component active efflux systems in *P. aeruginosa***

<table>
<thead>
<tr>
<th>Cytoplasmic membrane pump</th>
<th>Periplasmic linker</th>
<th>Outer membrane channel</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MexB</td>
<td>MexA</td>
<td>OprM</td>
<td>β-lactams including <strong>meropenem except imipenem</strong>, Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin,</td>
</tr>
<tr>
<td>MexD</td>
<td>MexC</td>
<td>OprJ</td>
<td><strong>Meropenem</strong>, Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, penicillins except carbenicillin and sulbamicillin, cefepime, cefpirome</td>
</tr>
<tr>
<td>MexF</td>
<td>MexE</td>
<td>OprN</td>
<td><strong>Carbapenems</strong>, Fluoroquinolones,</td>
</tr>
<tr>
<td>MexY</td>
<td>MexX</td>
<td>OprM</td>
<td><strong>Meropenem</strong>, Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, aminoglycosides, penicillins except carbenicillin and sulbamicillin, cefepime, cefpirome</td>
</tr>
</tbody>
</table>

3.5.1.3. Porin defects in *P. aeruginosa*

Loss of the OprD porin in *P. aeruginosa* is an important mechanism associated with imipenem resistance in this pathogen [Queenan *et al.*2007]. *P. aeruginosa* isolates become imipenem resistant is via mutational loss of a 54-kDa OMP, Opr D. The *P. aeruginosa* porin Opr D is a substrate-specific porin that has been shown to facilitate the diffusion of basic amino acids, small peptides that contain
these amino acids, and carbapenems into the cell. This aqueous porin shares close homology to the nonspecific porin Omp F in E. coli. Loss of Opr D is associated with resistance to imipenem and reduced susceptibility to meropenem. Loss of Opr D production is likely due to inactivation of the Opr D gene. Loss of Opr D does not confer resistance to \(\beta\)-lactams other than the carbapenems. Mutational loss of Opr D is frequent during imipenem therapy as demonstrated in clinical studies. The impact of Opr D deficiency on the potency of these carbapenems does not always push the MICs above the susceptible breakpoint, and additional resistance mechanisms (efflux pump and/or carbapenemase) may be required to provide resistance to the carbapenems.[Livermore 2000, 2002, Bonomo, 2006, Strateva T et al 2009]

### 3.5.2. Carbapenem resistance in Acinetobacter species

*Acinetobacter baumannii* is the most common species associated with infections in the health care settings. Less common species isolated in clinical microbiology laboratory are *Acinetobacter lwoffii, Acinetobacter johnsoni* and *Acinetobacter junii*. Multidrug resistant *A. baumannii* cause a multitude of infections that include bacteremia, pneumonia, meningitis, urinary tract and wound infections. Their ability to survive under a wide range of environmental conditions make them a frequent cause of outbreaks of infection and an endemic health care associated pathogen. *A. baumannii* are often resistant to a wide variety of antimicrobial agents, including carbapenems. Carbapenem resistance in *A. baumannii* is due to a variety of combined mechanisms such as hydrolysis by \(\beta\) lactamases, alterations in outer membrane protein and penicillin binding proteins and increased activity of efflux pumps. [Peleg et al 2008]
3.5.2.1. Carbapenemases in *Acinetobacter* spp.

Of the β-lactamases, those with carbapenemase activity are most concerning and include the serine oxacillinases (Ambler class D OXA type) and the MBLs (Ambler class B). These carbapenemases are of greatest concern as they are encoded by genes which are transmissible. The Ambler class A carbapenemases KPC has been reported in Puerto Rico. (Table-3) Thus far, the Ambler class A carbapenemases - GES, SME, NMC, and IMI have not been described for *A. baumannii*. [Maragakis *et al.* 2008, Peleg *et al.* 2008, Poirel *et al.* 2006].

**Table-3: Acquired carbapenemases identified in *A. baumannii***

<table>
<thead>
<tr>
<th>MBL</th>
<th>Ambler class</th>
<th>Plasmid/ chromosomal</th>
<th>Geographical origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP-1</td>
<td>B</td>
<td>Plasmid</td>
<td>Italy, Japan, South Korea</td>
</tr>
<tr>
<td>IMP-2</td>
<td>B</td>
<td>Plasmid</td>
<td>Italy, Japan</td>
</tr>
<tr>
<td>IMP-4</td>
<td>B</td>
<td>?</td>
<td>Hong Kong</td>
</tr>
<tr>
<td>IMP-5</td>
<td>B</td>
<td>?</td>
<td>Portugal</td>
</tr>
<tr>
<td>IMP-6</td>
<td>B</td>
<td>?</td>
<td>Brazil</td>
</tr>
<tr>
<td>IMP-11</td>
<td>B</td>
<td>?</td>
<td>Japan</td>
</tr>
<tr>
<td>VIM-2</td>
<td>B</td>
<td>Plasmid</td>
<td>South Korea</td>
</tr>
<tr>
<td>SIM-1</td>
<td>B</td>
<td>?</td>
<td>South Korea</td>
</tr>
<tr>
<td>OXA-23</td>
<td>D</td>
<td>Plasmid</td>
<td>UK, French Polynesia, Brazil, Iraq</td>
</tr>
<tr>
<td>OXA-24</td>
<td>D</td>
<td>Chromosomal</td>
<td>Spain</td>
</tr>
<tr>
<td>OXA-25</td>
<td>D</td>
<td>Chromosomal</td>
<td>Spain</td>
</tr>
<tr>
<td>OXA-26</td>
<td>D</td>
<td>Chromosomal</td>
<td>Spain</td>
</tr>
<tr>
<td>OXA-27</td>
<td>D</td>
<td>?</td>
<td>Singapore</td>
</tr>
<tr>
<td>OXA-40</td>
<td>D</td>
<td>Chromosomal</td>
<td>France, Spain, Portugal</td>
</tr>
<tr>
<td>OXA-58</td>
<td>D</td>
<td>Plasmid/ Chromosomal</td>
<td>France, Spain, Italy, Greece, UK, Austria, Romania, Iraq, Argentina, Kuwait</td>
</tr>
</tbody>
</table>

**KPC in *Acinetobacter* species:** The first report of multidrug-resistant *A. calcoaceticus-baumannii* complex clinical isolates harboring the KPC gene was from Puerto Rico in 2010. From a total of 274 multidrug-resistant *Acinetobacter* isolates collected from 17 hospitals, 10 (3.4%) were identified as KPC positive.
Sequencing of $\text{bla}_{\text{KPC}}$ detected the following variants: KPC-3 in seven isolates; KPC-4, KPC-2, and a novel KPC-10 in one isolate each. The presence of the $\text{bla}_{\text{KPC}}$ gene in Acinetobacter species adds another important element to an organism already harboring multiple innate and acquired mechanisms of resistance with the real possibility of horizontal transfer of a very troublesome and potent carbapenemase associated with transposons which can be transferred from one bacterium to another. [Robledo et al. 2010, 2011]

**Class B Metallobetalactamase in Acinetobacter species:** IMP-like, VIM-like, SIM-1 and NDM are the MBLs identified in A. baumannii. The IMP and VIM variants confer a high level of carbapenem resistance in A. baumannii. Six IMP variants belonging to three different phylogroups have been identified in A. baumannii, namely IMP-1 in Italy, Japan and South Korea; IMP-2 in Italy and Japan; IMP-4 in Hong Kong; IMP-5 in Portugal; IMP-6 in Brazil; and IMP-11 in Japan. In addition, IMP-4 has been identified in an Acinetobacter junii clinical isolate from Australia. VIM enzymes have been identified very rarely in A. baumannii, being represented only by VIM-2 reported in South Korea. [Poirel L et al. 2006, Peleg et al. 2008]. SIM-1 has been reported only in A. baumannii from South Korea. The enzyme SIM-1 (for “Seoul imipenemase”) has the closest amino acid identity to the IMP family (64 to 69%). SIM-1 was discovered in a large-scale screen of imipenem-resistant Acinetobacter sp. isolates in Korea. [Lee et al. 2005].

**NDM-1 in Acinetobacter species:** Pfeifer et al. detected NDM-1 in A. baumannii isolated from a patient repatriated to Germany from Serbia in 2007 [Pfeifer et al. 2011]. Importation of NDM-1-producing A. baumannii strain from Serbia has also been described by Poirel et al. [Poirel et al. 2012b] Other A. baumannii isolates expressing NDM-1 MBL have been isolated in China, Belgium and India [Chen et al.
2011b, Bogaerts et al.2012, Karthikeyan et al.2010]. It is remarkable that \( \text{bla}_{\text{NDM-1}} \) was also found on a plasmid in \( A. \text{lwoffii} \) in China [Hu Y et al 2011]. The new NDM-2 variant was first detected in \( A. \text{baumannii} \) from a patient transferred from Egypt to Germany [Kaase et al. 2011]. Recently, clonal spread of NDM-2 producing \( A. \text{baumannii} \) strains have been described in a rehabilitation ward in Israel and in the United Arab Emirates [Espinal et al. 2011,Ghazawi et al.2012].

**OXA Carbapenemases in *Acinetobacter* species:** The first identified OXA type enzyme with carbapenem hydrolysing activity was from \( A.baumannii \) strain isolated in 1985 from Scotland and was originally named \( \text{ARI-1}(\text{Acinetobacter Resistant to Imipenem}), \) but was renamed as \( \text{bla}_{\text{OXA-23}}. \text{Bla}_{\text{OXA-23}} \) cluster (\( \text{bla}_{\text{OXA-23,27,49}} \)) now contribute to carbapenem resistance in \( A. \text{baumannii} \) globally. This plasmid-encoded enzyme has been discovered in England, Brazil, Polynesia, Singapore, Korea and China. Two other plasmid encoded acquired OXA type clusters with carbapenamase activity have been described, including and \( \text{bla}_{\text{OXA-58}} \)-like genes and \( \text{bla}_{\text{OXA-24}} \)-like (\( \text{bla}_{\text{OXA-24, 25,26,40}} \)). OXA-58, a plasmid-borne carbapenemase, was found in France, England, Argentina, Spain, Turkey, Romania, Austria, Greece, Scotland, and Kuwait. \( \text{Bla}_{\text{OXA-24}} \) was initially reported from Spain. The \( \text{bla}_{\text{OXA-23}}, \text{bla}_{\text{OXA-24}} \) and \( \text{bla}_{\text{OXA-58}} \) like enzymes are plasmid / chromosomally encoded which explains their widespread distribution.[Maragakis et al.2008, Peleg et al 2008, Perez et al 2007, Walther-Rasmussen et al 2006]

The \( \text{bla}_{\text{OXA-51}} \)-like gene cluster is unique in that it is naturally occurring in \( A. \text{baumannii} \), hence its chromosomal location and prevalence. Similar to other class D enzymes, they have greater affinity for imipenem than meropenem. Their role in carbapenem resistance is related to the presence of an insertion sequence \( \text{ISA}_{\text{Bal1}}, \) situated upstream possibly providing a promoter for hyper production of beta
lactamase genes. [Susan et al. 2006, Koh et al. 2007]. Strains that harbour more than one OXA encoding genes have been reported from a variety of geographical areas [Yang et al. 2009, Feizabadi et al. 2008, Mendes et al. 2009]

3.5.2.2. Non Enzymatic mechanisms in Acinetobacter species:

β-Lactam resistance, including carbapenem resistance, has also been ascribed to non-enzymatic mechanisms, including changes in outer membrane proteins, multidrug efflux pumps, and alterations in the affinity or expression of penicillin-binding proteins. [Peleg et al. 2008]

**Outer membrane proteins:** A. baumannii possesses OMPs that play a role in carbapenem resistance. By reduction of transport into the periplasmic space via changes in porins or OMPs, access to PBP is reduced. With less β-lactam entering the periplasmic space, the weak enzymatic activity of the β-lactamase is amplified. Many outbreaks of infection with imipenem-resistant A. baumannii are due to porin loss. Quale et al. found that carbapenem-resistant isolates of A. baumannii had reduced expression of 47-, 44-, and 37-kDa OMPs. [Quale et al. 2003, Bonoma et al. 2006]. In 2002, Limansky et al. demonstrated that imipenem resistance was associated with the loss of a 29-kDa OMP in clinical isolates of A. baumannii in which no carbapenemase activity had been detected. [Limansky et al. 2002]. Similarly, resistance to imipenem and meropenem in multidrug-resistant A. baumannii clinical isolates has also been associated with loss of a heat-modifiable 29-kDa OMP, designated Car O [Mussi et al. 2005]. Carbapenemase production and decreased permeability, is responsible jointly for the high-level carbapenem resistance observed. [Bou et al. 2000]. Another interesting observation was made by Del Mar Tomas et al. who studied an A. baumannii isolate exhibited high-level resistance to carbapenems due to the loss of a 31- to 36-kDa OMP. [Del Mar Tomas et al. 2005]
**Efflux pump in Acinetobacter species:** The resistance-nodulation-division (RND) family-type pump AdeABC is the best studied thus far and has a substrate profile that includes β-lactams (including carbapenems), aminoglycosides, erythromycin, chloramphenicol, tetracyclines, fluoroquinolones, trimethoprim, and ethidium bromide [Peleg *et al.* 2008, Limansky *et al.* 2002]. Similar to other RND-type pumps, AdeABC has a three-component structure: AdeB forms the transmembrane component, AdeA forms the inner membrane fusion protein, and AdeC forms the OMP. AdeABC is chromosomally encoded and is normally regulated by a two-component system with a sensor kinase (AdeS) and its associated response regulator (AdeR). Point mutations within this regulatory system have been associated with pump overexpression [Marchand *et al.* 2004, Nemec *et al.* 2007, Ruzin *et al.* 2007].

**PBP in Acinetobacter species:** Modification of PBPs as a source of imipenem resistance in *A. baumannii* has been investigated only rarely. Gehrlein *et al.* studied a carbapenem-susceptible isolate and a derived resistant mutant obtained in vitro, and showed that the resistant mutant hyper-produced a 24-kDa PBP. Another study described the existence of 12 PBP patterns among a collection of *A. baumannii* isolates with variable β-lactam resistance profiles. [Gehrlein *et al.* 1991, Fernandez-Cuenca *et al.* 2003]

### 3.6. Detection of carbapenemases in GNB

Adequate detection of carbapenemase-producing GNB is crucial for infection control measures and appropriate choice of antimicrobial therapy. The production of a given carbapenemase may confer a particular β-lactam resistance phenotype, depending on the bacterial species, the expression level, the enzyme type or variant, and the presence of additional resistance mechanisms such as permeability reduction and/or efflux and/or activity of other β-lactamases.
A significantly elevated MIC or a decreased inhibition zone with disk diffusion testing) of a carbapenem should make a clinical isolate eligible for further testing for carbapenemase production by means of specific methods. However, carbapenem MICs for carbapenemase producers may vary within a broad range of values, and even lay within the susceptibility range, as defined by either the current Clinical Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints. Indeed, such low levels of resistance to carbapenems have often been observed in Enterobacteriaceae producing carbapenemases of different types.

In the case of Enterobacteriaceae, it is now suggested that carbapenemase-detecting phenotypic tests should be performed in isolates exhibiting even a small reduction of susceptibility to carbapenems, including ertapenem. The CLSI recommends screening for isolates with intermediate or resistance to one or more carbapenems and resistance to at least one cephalosporin of subclass III (ceftotaxime, ceftriaxone or ceftazidime) for possible carbapenemase production in Enterobacteriaceae. [CLSI 2011,2012,2013]

Carbapenem susceptibility testing is done by the standard methods such as disc diffusion method and determination of MIC by agar/broth dilution methods. Special care should be applied in preparing bacterial inocula. The broth microdilution and disk diffusion methods are considered to be more reliable for the detection of all types of carbapenemase-mediated resistance. [CLSI 2011, Stuart et al.2010, Mirigou et al. 2010, Castenheira et al.2004, Lee et al.2005, Queenan et al. 2007].
3.6.1. Phenotypic tests for detection of carbapenemase production

A number of simple phenotypic tests, most of them in the disk diffusion format, have been described and evaluated as methodologies for the specific detection of carbapenem resistant organisms. Phenotypic confirmation may be performed using one or two methods, the Modified Hodge Test (MHT) and the carbapenemase inhibition tests. MHT is used for detection of diffusible carbapenemases, and the inhibition tests are used to distinguish between the different classes of carbapenemases. [Stuart et al. 2010, Mirigou et al. 2010, Nordmann et al. 2012c]

3.6.1.1. Modified Hodge test:

MHT or the clover leaf method has been extensively used as a general phenotypic method for the detection of carbapenemase activity, and it has been the only method recommended by the CLSI for detection of carbapenemase in Enterobacteriaceae. The test is based on the inactivation of a carbapenem by either whole cells or cell extracts of the test organisms, which enables a carbapenem-susceptible indicator strain to extend growth towards a carbapenem disk, along the streak of inoculum of the test strain. The assay is, overall, sensitive for the detection of a carbapenemase-mediated mechanism of resistance to carbapenems but does not provide information regarding the type of carbapenemase involved. [Pasteran et al. 2009]. Some investigators have raised the problem of difficulties in the interpretation of the clover leaf test for weak carbapenemase producers, particularly for MBLs in Enterobacteriaceae. In the case of MBL producers, it has been suggested that addition of zinc sulphate may improve the MHT performance. [Mirigou et al. 2010]. Disadvantages of this test are possible interpretation difficulties and the fact that different classes of carbapenemases cannot be distinguished. Specificity may be low
because CTX-M ESBL- or AmpC-producing isolates with reduced or absent porin expression may give false-positive results. [Stuart et al. 2010, Mirigou et al. 2010]

3.6.1.2. Inhibition of carbapenemase activity (synergy tests)

Detection of class A carbapenemases: Specific phenotypic assays for the identification class A carbapenemases producing strains, are based on the inhibitory effect of boronic acids, usually 3-aminophenylboronic acid (APB). Of several indicator β-lactams tested, either meropenem or imipenem were pointed out as the preferable compounds. The cut-off values of zone diameter differences between disks with a carbapenem plus APB and the carbapenem alone were proposed as being indicative of production of class A carbapenemase (≥4 to ≥7 mm). With the use of meropenem disks, with or without 400 µg of APB, the specificity in diagnosing KPC-producing K. pneumoniae isolates and differentiating them from plasmid Amp C-producing K. pneumoniae and E. coli proved excellent. Apart from the disk diffusion approaches, a method has been developed in which MICs of carbapenems are evaluated both in the absence and in the presence of APB (0.3 g/L) by agar dilution. A three-fold or greater reduction of carbapenem MIC in the presence of APB has been proposed as the cut-off value for positive isolates. In general, boronic acid-based methods exhibit high sensitivity in the detection of KPC producers, which makes these methods very promising. [Pasteran et al. 2009, Tsakris et al. 2009]

Detection of MBLs: Several inhibitor-based tests have been developed for the specific detection of MBL producers. These are based on the synergy between MBL inhibitors—such as EDTA, EDTA plus 1,10-phenanthroline, thiol compounds (2-mercaptopropionic acid or sodium mercaptoacetic acid) and dipicolinic acid—and a carbapenem (imipenem and/or meropenem) and/or an oxyimino-cephalosporin (ceftazidime) as indicator β-lactam compounds. These tests take advantage of the
metalloenzyme dependence on zinc ions, and use the chelating agents to inhibit β-lactam hydrolysis. Various formats (disk diffusion or broth dilution) of EDTA-based synergy tests have been the most commonly used and evaluated. [Lee et al. 2001, 2003, Hemalatha et al. 2005, Yong et al. 2002, Migliavacca et al. 2002, Arakawa et al. 2000]. The double-disk synergy test (DDST) and the combined disk test, using different amounts of EDTA and, in the case of DDST, different distances between the disks, exhibit high sensitivity even with isolates with low carbapenem resistance levels. It has been suggested that zinc supplementation of the culture medium may increase the sensitivity of the method. The E-test MBL strip, based on synergy between EDTA and imipenem, has been credited with good sensitivity and specificity for detection of MBL-producing organisms although it has been repeatedly pointed out that its specificity might be impaired by, the possible intrinsic activity of EDTA. Despite the good performance of inhibitor tests in the detection of MBLs in general, it should be remembered that MBL inhibitors act non-specifically and affect other structures and processes (e.g. outer membrane permeability). The results should be interpreted cautiously, and it is strongly recommended to have them confirmed by genotypic methods. All inhibitor-based synergy tests should include a control for the intrinsic activity of the inhibitor. [Mirigou et al. 2010]

**Class D carbapenamases:** There is no specific phenotypic test to detect Class D carbapenemases in the laboratory. PCR based methods remain the gold standard for their detection. [Poirel et al. 2010a]

**3.6.2. Detection of carbapenemase encoding genes by molecular methods**

Molecular methods such as simplex and multiplex PCRs, real-time PCR, DNA hybridization and sequencing have been commonly used for the identification of carbapenemase genes in research laboratories and reference centers. Nowadays, some
of these methods, mostly PCR, are routinely performed in some clinical laboratories in order to circumvent the problems of the phenotypic tests. [Hong et al. 2012, Nordmann et al. 2011a, Monteiro et al. 2012, Poirel et al. 2011d, Dallenne et al. 2010, Castanheira et al. 2011a]

3.6.3. Other methods

Spectrophotometric measurement of carbapenem hydrolysis is considered to be the reference standard method for detection of carbapenemase production in a suspected CP organism. Hydrolysis of carbapenems in the presence or absence of inhibitors (i.e. EDTA for MBLs, tazobactam or clavulanic acid for KPCs, NaCl for most OXA), performed with crude cell extracts or partially purified enzymes, provide additional information concerning the enzyme type. These laborious and technically demanding assays are performed in reference laboratories.[Mirigou et al. 2010]

Isoelectric focusing (IEF) separates proteins by charge, and detection of β-lactamases is accomplished with the chromogenic cephalosporin nitrocefin. Although IEF results cannot identify a specific β-lactamase, information about isoelectric point and inhibition characteristics can be obtained by this method. IEF 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 [Queenan et al. 2007]

3.7. Treatment of infections caused by Carbapenem resistant GNB.

Of the 6 famous ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter species, Pseudomonas aeruginosa, and Enterobacter species) recognized as the most important emerging threats of this
century, 4 are gram-negative bacilli (*K* *pneumoniae*, *Acinetobacter* species, *P* *aeruginosa*, and *Enterobacter* species)[Kanj *et al*. 2011]

Recognizing carbapenemase expression is the key to the appropriate management of infections caused by carbapenem-resistant GNB. Unusually elevated MICs to carbapenems should arouse suspicion for a carbapenem-resistant isolate and preclude the use of carbapenems even if the MICs do not exceed the breakpoints for resistance. As with ESBL-producing organisms, carbapenemase-producing strains are likely to exhibit simultaneous resistance to aminoglycosides and fluoroquinolones.

**Antibiotic Options**

**Aztreonam**: It is stable to metallo-carbapenemases, including IMP, VIM and NDM. However, for isolates that also co-produce AmpC or ESBL, aztreonam is ineffective. [Karthika *et al*. 2009, Walsh *et al*. 2005]

**Sulbactam**: Sulbactam is active against *A.baumannii* by inhibiting PBP-2. In most countries it is available as a co-formulation with ampicillin. Sulbactam is useful in the treatment of carbapenem resistant *A.baumannii* infections in combination with colistin. [Kosmidis *et al*. 2012].

**Tigecycline**: Tigecycline, a tetracycline analogue is the first glycylicycline to be launched for clinical use. It acts by inhibiting the protein synthesis in the bacterial cell by binding to the 30S subunit of the ribosome. It evades the Tet(A-E) efflux pumps, which account for most acquired resistance to tetracycline and minocycline in Enterobacteriaceae and *Acinetobacter* spp. Its capacity to penetrate into various tissues makes it useful in the treatment of infections of the skin and soft tissues as well as intra-abdominal infections, whereas its low serum concentrations compromise its use in bloodstream infections. It is not useful in treatment of nosocomial pneumonia as indicated by poor results in the study of ventilator associated
pneumonia. Despite tigecycline being one of the first-line agents for use in the setting of carbapenemase-producing isolates, clinical failures have been reported in the literature, including ESBL- and KPC-producing isolates, which had a negative clinical and/or microbiological outcome with tigecycline. In addition, it is affected by the intrinsic multidrug pumps of *P. aeruginosa* and Proteae and therefore it is not useful to treat infections caused by them.[Kanj et al.2011]

**Polymyxin:** Given limited therapeutic options, clinicians have returned to the use of polymyxin B or polymyxin E (colistin) for the most carbapenem resistant gram negative infections. Polymyxin B differs from colistin by only one aminoacid. These drugs acts by disturbing the bacterial cell membrane, thus increasing permeability, leading to cell death. Although colistin retained activity against carbapenemase-producing Enterobacteriaceae in initial studies, more recent data suggest that resistance to colistin is emerging, and outbreaks of colistin-resistant strains have been reported.[Kanj et al.2011, Peleg et al. 2008, Gupta et al.2011]

**Fosfomycin:** Fosfomycin inhibits bacterial cell wall synthesis, thereby exhibiting bactericidal activity against gram-positive and gram-negative pathogens. Fosfomycin is useful for the treatment of uncomplicated urinary tract infections at a single oral dose. The emergence of resistance among GNB has sparked new interest in using fosfomycin to treat infections caused by MDR isolates.[Kanj et al.2011]

**Rifampin:** In vitro studies suggest that rifampin has a synergistic activity when used as part of a combination therapy regimen against carbapenemase-producing *E coli, K pneumoniae* and *A. baumannii*. [Kanj et al.2011]

**Agents Under Development.** Agents under development include new β-lactamase inhibitors with activity against carbapenemases, such as MK-7655, NXL104, and 6-
alkylidenepenam sulfones, and several bis-indole compounds, the mode of action of which is currently unidentified.[Kanj et al.2011]

**Extended-Infusion Strategy for β-Lactams**: Carbapenems have also been evaluated in extended-infusion regimens. Lengthening meropenem infusions from 30 minutes to 3 hours was found to be advantageous with isolates of *P. aeruginosa* and *Acinetobacter* species with intermediate resistance. This benefit was not observed with resistant isolates having very high MICs.[Peleg et al. 2008, Kanj et al. 2011, Manchanda et al. 2010, Chambers et al. 2005]

### 3.8. Epidemiology of carbapenem resistance in Gram negative bacteria

Studies illustrating the prevalence of resistance among Gram negative bacteria are essential because wide regional differences exists, accentuating the need to take into account the local epidemiology (at the level of the country, the region, the hospital, and at times the individual hospital units/wards) when making decisions about empirical therapy for serious infections.

#### 3.8.1. Epidemiology of Carbapenem resistance in Enterobacteriaceae

Carbapenem resistant Enterobacteriaceae (CRE) appear to have been uncommon in the United States before 1992. Using data from the National Nosocomial Infection Surveillance (NNIS) system from 1986 to 1990, it was reported that only 2.3% of 1825 *Enterobacter* isolates tested non-susceptible to imipenem. However, over the last decade CRE have been reported more commonly. In the Meropenem Yearly Susceptibility Test Information Collection Program (MYSTIC), meropenem resistance among clinical isolates of *K. pneumoniae* increased significantly from 0.6% in 2004 to 5.6% in 2008. Among isolates reported to the National Healthcare Safety Network (NHSN) in 2006–2007, carbapenem resistance
was reported in up to 4.0% of *E. coli* and 10.8% of *K. pneumoniae* isolates that were associated with certain device-related infections.[Gupta et al.2011]. The Center for Disease Control and Prevention (CDC) reported that, among health care–associated infections, 8% of *Klebsiella* spp. isolates were carbapenem resistant in 2007 compared with <1% in 2000 [Sanchez et al.2013]

**Enterobacteriaceae producing KPC**

KPC producing *Enterobacteriaceae* were first reported in a clinical specimen from a patient in North Carolina in 2001. Subsequently, outbreaks and transmission of KPC-producing organisms were reported, predominantly from the northeastern United States. Surveillance studies suggested that the epicenter of this epidemic was the state of New York. Later, isolates producing KPC-2 and KPC-3 (a point mutant of KPC-2) became established in hospitals in neighboring states, apparently due to transfer of colonized patients. During the same period, KPC-producing *K. pneumoniae* also emerged in Latin America and Israel. In Greece, KPC-positive *K. pneumoniae* became dominant in tertiary care hospitals, reaching epidemic proportions in a matter of approximately 2 years. In Northern and Western European countries, KPC prevalence remains low. Nevertheless, a multihospital outbreak has already occurred in France. Higher prevalence have been reported from Poland and Italy, where KPC producers appear to be established in various regions. The rapid global dissemination of KPC producing *K. pneumoniae* implies multiple transmission routes. According to a widely held scenario, an important event was the introduction of KPC-positive *K. pneumoniae* from the United States to Israel, followed by spread to neighboring countries and via Greece to other European countries. KPC-producing isolates of various other enterobacterial species, including *E. coli* and *E. cloacae*, have been reported in settings where the prevalence of KPC-positive *K. pneumoniae* is
high. Outbreaks of KPC-producing *E. coli* have occurred in health care facilities in various countries. [Gupta *et al.* 2011].

**Enterobacteriaceae producing MBL**

MBLs are mostly of VIM- and IMP-types, but the recently emerged NDM-type is becoming the most threatening carbapenemase. MBL enzymes are found worldwide and like the KPCs have spread rapidly (especially NDM-1). Described in 2008 and retrospectively found in isolates collected in 2006, the NDM-producing Enterobacteriaceae are now the focus of worldwide attention because of high-level carbapenem and their rapid global spread, some of which has been facilitated by extensive international travels. [Hara *et al.* 2013].

*K. pneumoniae* strains producing enzymes belonging to any of the three MBL families (VIM, IMP, and NDM) have already achieved international spread, though significant local differences do exist. VIM-positive *K. pneumoniae* was first observed around 2001 to 2003 in Southern Europe and was introduced later to Northern Europe (e.g., Germany, France, and the Scandinavian countries) and the United States, mostly through colonized patients transferred from high-prevalence areas. Isolation rates of VIM positive *K. pneumoniae* in Northern Europe and the United States remain low, though some infection clusters limited to single hospitals have been reported. In addition, sporadic cases have been recorded in Tunisia, South Korea, and Venezuela. Until recently, VIM-producing *K. pneumoniae* and other enterobacteria were frequently isolated in Mediterranean countries, reaching epidemic proportions only in Greece.

Acquisition of IMP MBLs by *K. pneumoniae* was described during the 1990s, primarily in Japan, as well as in Taiwan and Singapore. IMP-positive *K. pneumoniae* clinical isolates remain frequent in Japan. IMP-4-producing *K. pneumoniae* strains
have also caused hospital outbreaks in China and Australia. Dissemination of IMP producing Enterobacteriaceae in the rest of the world appears to be limited, with single cases identified in Turkey, Lebanon, Brazil, and the United States. As usual, limitations and differences in surveillance systems in different countries inevitably affect the reliability and comparability of international epidemiological data on IMP (or indeed VIM)-positive K. pneumoniae. [Gupta et al. 2011, Tzouvelekis et al. 2012].

In stark contrast, the results of the internationally concerted effort and resources allocated for the elucidation of the transmission routes and public health impact of enterobacteria, mainly E. coli and K. pneumoniae strains producing NDM, the most recently identified MBL type, were spectacular. These efforts produced a wealth of data regarding the epidemiology of NDM producers. Furthermore, the bla NDM genes have spread to various enterobacterial species other than K. pneumoniae and E. coli. A characteristic of NDM-producing K. pneumoniae isolates has so far been their rapid dissemination; indeed, infected or colonized humans without obvious connection to the Indian epidemic are increasingly being reported in several countries. [Gupta et al. 2011].

**Enterobacteriaceae producing OXA-48**

OXA-48-producing K. pneumoniae was first detected sporadically in Turkey, in 2001. Hospital outbreaks in the main cities of this country soon followed. About the same time, OXA-48-positive K. pneumoniae isolates were also identified in other Middle Eastern and North African countries as well as in Western European countries, including the United Kingdom, Belgium, France, Germany, and the Netherlands. Emergence of OXA-48 producers in the latter countries has been attributed mainly to colonized patients transferred from North Africa. Recently, an
important outbreak due to an OXA-48-producing *K. pneumoniae* strain was reported in a Dutch hospital. However, there are no indications of an overall significant spread of these microorganisms across Europe. Although the Middle East and North Africa remain the main foci of infection, the recent isolation of *K. pneumoniae* isolates producing OXA-48-type enzymes in India, Senegal, and Argentina suggests an expansion that can safely be considered global. Additionally, the recent isolation of OXA-48 producers belonging to species other than *K. pneumoniae* underlines the spreading potential of *bla* \( \text{OXA-48} \).

### 3.8.2. Epidemiology of carbapenem resistance in *A. baumannii*

Surveillance studies indicate that the percentage of carbapenem-resistant *Acinetobacter* species gradually increased over the last ten years in Europe, North America, Asia and Latin America. Numerous outbreaks of carbapenem-resistant *A. baumannii* were reported from hospitals in Northern and Southern Europe and the Middle East, North America and Latin America, Tunisia and South Africa, China, Taiwan, Singapore, Hong Kong, Japan, South Korea and Australia. In the majority of cases, one or two epidemic strains were detected in a given hospital. Transmission of such strains was observed between hospitals in the same city and also on a national scale and a direct epidemiological link was established in several cases. The inter-hospital transfer of colonised patients was demonstrated during multi-facility outbreaks that occurred in The Netherlands, Italy, South Africa, and Tunisia. The international transfer of patients colonised by carbapenem-resistant *A. baumannii* was also reported. More recently, several cases of United Kingdom and US military and nonmilitary personnel returning from operations in Iraq and Afghanistan and harbouring infections caused by carbapenem-resistant *A. baumannii* were reported.
Outbreaks caused by carbapenem-resistant \textit{A.baumannii} have also been observed in developing countries such as Morocco, Thailand, India, and Indonesia.

In a report from a teaching hospital in Spain (2002), the prevalence of imipenem-resistant \textit{Acinetobacter} spp. had increased from no resistance in 1991 to 50\% in 2001. Among \textit{Acinetobacter} spp. derived from 30 European centers from the worldwide collection of SENTRY from 2001 to 2004, the proportion of strains resistant to imipenem, meropenem was: 26.3 and 29.6 \%, respectively. Gladstone \textit{et al.} from Vellore, India (2005), reported a prevalence of 14\% carbapenem-resistant \textit{Acinetobacter} spp., isolated from tracheal aspirates (56). In Delhi, India (2006), the prevalence of carbapenem resistance in \textit{Acinetobacter} spp. isolated from different clinical samples was found to be almost 35\%. In Greece, the proportion of imipenem-resistant \textit{A. baumannii} isolates from patients hospitalized between 1996 and 2007, in tertiary care hospitals, in several regions of the country rose from no resistance to 85\% (ICUs), 60\% (medical wards), and 59\% (surgical wards) [Greek System for Surveillance of Antimicrobial Resistance (GSSAR)..The prevalence of imipenem resistance in \textit{Acinetobacter baumannii} isolated from a burns unit of USA (2007) was found to be as high as 87\% [Manchandra \textit{et al.} 2010, Zarrilli \textit{et al.} 2009, Gladstone \textit{et al} 2005.]

3.8.3. Epidemiology of carbapenem resistance in \textit{P.aeruginosa}

\textit{P.aeruginosa} has evolving virulence characteristics and antimicrobial resistance patterns which make it a difficult target for antibiotic therapy. It was the most frequently found bacterium in lower airway infections in Brazilian hospitals of the SENTRY study, displaying a resistance of 49\% to imipenem. The MYSTIC program carried out in 20 Brazilian hospitals also found that \textit{P. aeruginosa} was the
most frequent Gram negative isolated from nosocomial infections, with resistance
rates of 64% to meropenem. The USA’s NNIS showed that in 2003 among ICU
patients of 17.7%, of isolates resistance to imipenem.[Millena et al.2008]. In
Lithuania, the resistance of P. aeruginosa to carbapenems differed: 23.9% of strains
were resistant to imipenem, 11.3% – to meropenem. During European MYSTIC
study, 31.8% of P. aeruginosa strains resistant to carbapenems were obtained, 7.0%
in USA, 54.3 in Turkey, 6.7% in UK. Russian study showed that 22.9% of P. aeruginosa strains were resistant to imipenem. According to the data of the SENTRY
program, 5.1–8.4% of P. aeruginosa strains obtained in Canada were resistant to
meropenem, 10.2–26.2% in Europe, and 7.6–9.0% in USA. [Greta Gailienè 2007,
Gales et al. 2002, Mutnick et al.2002, Garcia-Rodriguez 2002]. The prevalence of
imipenem- resistant P. aeruginosa in Tehran has been reported to be within the range
of 16% to 100%. Shahid et al. found 100% MDR in P. aeruginosa burn isolates in
India in 2003.[Shahid et al. 2012]. In 2004, Ozkurt et al. reported 69.92% imipenem
resistance in P. aeruginosa burn isolates in Turkey.[Ozkurt et al.2005]. In a study
conducted between 1996 and 1998 in Korea, 52% of P. aeruginosa were resistant to
imipenem. In another study performed in Karachi, Pakistan in 2003, imipenem
resistance in P. aeruginosa was 32.7%.[Moazami-Goudarzi et al. 2013]. The
evaluation of carbapenem resistance in P.aeruginosa strains in two Mexican hospitals
during 2005-2010 revealed 17.8% resistance to imipenem and 12.8% resistance to
meropenem.[Morfin-Otero et al. 2012]. During the years 2001 and 2006, rates of non-
susceptibility among P. aeruginosa isolates in Brooklyn, NY, ranged from 30 to 31%
for imipenem and 23% for meropenem [Lister et al. 2009 ] Rates of carbapenem
resistance in Arabian Peninsula over the last decade varied widely among the different
countries and revealed a rising trend: 10.4-19% in 1994-95 from Kuwait ; 22% in
1998 from Qatar; 4-9% between 2001-2004 from Saudi Arabia; 63.3% in 2004-2007 from Oman; 16.2-91% in 2009-2010 from Saudi Arabia.[Zowawi et al 2013]

3.9. Risk factors for infection with carbapenem resistant Gram negative bacteria and impact of infection

Identification of the risk factors associated with carbapenem resistant bacterial infections is necessary to guide appropriate empirical antibiotic therapy, thus, reducing unfavorable outcomes and morbidity. It is also important that the epidemiology of carbapenem resistance in Gram negative bacteria be understood to implement adequate infection control measures.

Patients with colonization often have a history of prolonged hospitalization or antimicrobial therapy. Residence in an ICU, particularly in the presence of other patients who are colonized with carbapenem resistant organisms, predisposes patients to colonization. It is particularly seen in patients who are intubated and in those who have multiple intravenous lines, monitoring devices, surgical drains, or indwelling urinary catheters. Invasive devices used to facilitate fluid monitoring, administer medications, and provide lifesaving support may also be sources of colonization. [Maragakis et al 2008, Gupta et al. 2011, Hirsch et al 2010]. The ability of organisms particularly Pseudomonas and Acinetobacter spp., to survive in the environment for several days, even in dry conditions on particles and dust, probably contributes to the development and persistence of outbreaks. [Maragakis et al. 2008, Hirsch et al. 2010].

Many case control studies have revealed that prior exposure to antimicrobial therapy (duration and number of classes) has been the most common risk factor. Carbapenems and third-generation cephalosporins are the most commonly implicated
antibiotics, followed by fluoroquinolones and aminoglycosides. The second most common risk factor is mechanical ventilation. Other risk factors include stay in an ICU, duration of stay in ICU and hospital, severity of the illness, malignant disease, history of chronic obstructive pulmonary disease, recent surgery and invasive procedures. Hence, patients with carbapenem resistant infections tend to be critically ill or have prolonged hospital stay and receive multiple antimicrobials prior to the isolation.[Manchanda et al.2010, Maragakis et al 2008, Hirsch. et al 2010, Gupta.et al.2011.]

Furthermore, studies on A. baumannii and P. aeruginosa outbreaks have revealed environmental contamination as an important risk factor in the causation of outbreaks. During the outbreaks, extensive contamination of the environment, including respirators and air samplers in the vicinity of the infected or colonized patients have been documented. These organisms have been recovered from bed linen, mattresses, pillows, bed curtains, and blankets in the immediate vicinity of infected patients. It has also been isolated from food (including hospital food), ventilator equipment, suctioning equipment, infusion pumps, stainless steel trolleys, pillows, mattresses, tap water, bed rails, humidifiers, soap dispensers, and other sources. Also, other fomites like door handles, telephone handles, tabletops, and so on have tested positive during outbreaks, probably contaminated by the hands of the staff. In some settings, one or more epidemic clones often coexist with the endemic strains, making it difficult to detect and control transmission. Compounding to the problem of the ease to survive in a hospital environment and increasing antibiotic resistance, is the ability of the organisms to form biofilms. [Marchaim et al 2007, Oteo et al.2007,Hirsch et al.2010]
3.10. Impact of infection with carbapenem resistant Bacteria

Impact of infection with carbapenem resistant Enterobacteriaceae: When outcomes for patients with carbapenem-resistant Enterobacteriaceae are compared with those for patients with carbapenem-susceptible Enterobacteriaceae, carbapenem resistance has been independently associated with an increase in mortality. Age, mechanical ventilation, malignancy, heart disease, and ICU stay have been associated with increased mortality among those with carbapenem-resistant Enterobacteriaceae infections, whereas removal of the focus of infection (eg, catheter removal, debridement, or drainage) was independently associated with survival.[Borer et al.2012, Gasink et al.2009, Hussein et al.2009, Chang et al. 2011, Gupta et al.2011]

Impact of carbapenem resistant Acinetobacter infection: Carbapenem-resistant Acinetobacter infection usually occurs in severely ill patients in the ICU, therefore the associated crude mortality rate is high. However, it is difficult, to determine the attributable mortality of these infections independent of patients' severe underlying illnesses. Crude mortality rates of 30 - 75% have been reported for nosocomial pneumonia caused by A. baumannii. However, it has also been seen that mortality resulting from A. baumannii infection relates to the underlying cardiopulmonary and immune status of the host rather than the inherent virulence of the organism. Patients who are very ill with multisystem disease have higher mortality and morbidity rates, which may be due to their underlying illness rather than the superimposed infection with Acinetobacter.

Some studies concluded that Acinetobacter infection or colonization is associated with increased mortality. Few other studies did not find Acinetobacter infection to be independently associated with increased mortality. This was explained that such infection is a marker of increased mortality in patients with severe
underlying illness but not an independent predictor of mortality. Multidrug-resistant \textit{Acinetobacter} infection significantly prolong the duration of ICU stay and the median duration of hospitalization. The impact on length of stay may depend on the type of infection and the extent of antimicrobial resistance. [Manchanda \textit{et al}. 2010, Lee \textit{et al}. 2004, Fournier 2006]

\textbf{Impact of carbapenem resistant} \textit{P. aeruginosa} infection: Carbapenem resistant \textit{P. aeruginosa} infection not only increase mortality, but it is also associated with increased patient morbidity. Aloush \textit{et al}. demonstrated that isolation of MDR \textit{P. aeruginosa} was associated with a higher incidence of surgery often to remove the source of infection (i.e., debridement, amputation and graft removal), when otherwise not controlled with medical therapy. In addition, patients with MDR infection undergo increased number of invasive procedures (i.e., bronchoscopy, tracheostomy or catheter implantation). Length of hospital stay is another marker frequently used for morbidity. Aloush \textit{et al}. found isolation of MDR \textit{P. aeruginosa} to be associated with increased length of stay when compared with matched controls. Similarly, it has also been reported that patients treated with inappropriate empirical therapy had a statistically longer length of stay than those who received appropriate empirical treatment. Mortality is significantly higher among patients with imipenem-resistant \textit{P. aeruginosa} infection/colonization than patients with imipenem-susceptible infection/colonization. [Aloush \textit{et al}. 2004, Hirsch \textit{et al}. 2010, Harris AD \textit{et al}. 2001, Wang \textit{et al}. 2006, Fortaleza \textit{et al}. 2006]

\textbf{3.11. Infection prevention measures}

Although antimicrobial development efforts remain a cornerstone of CRE response efforts, interventions aimed at preventing the transmission of, and infections with, these organisms are also important. Delaying the emergence of carbapenem
resistance, particularly in areas where this resistance is still uncommon, can decrease the impact of these organisms as we await additional treatment options. CDC’s recommendations for preventing transmission of carbapenem resistant bacteria includes

1. **Laboratory detection of carbapenem detection**: Accurate detection of carbapenem resistance is the first step in prevention.

2. **Recognizing carbapenem resistance cases**: It is important for health care facilities to understand how common is carbapenem resistance in their institutions. In investigations conducted by the CDC, failure to recognize carbapenem resistant infections when they first occur in a facility has resulted in a missed opportunity to intervene before these organisms are transmitted more widely. This omission is often related to 2 issues: first, a failure to recognize an epidemiologically important organism that requires specific attention, and second, the lack of an established communication mechanism between infection-prevention personnel and the clinical laboratory. Based on current recommendations for the control of multidrug-resistant organisms (MDROs), the CDC recommends that, in areas where carbapenem resistant organisms are not endemic, acute care facilities review microbiology records for the preceding 6–12 months to determine whether carbapenem resistant organisms have been isolated at the facility if previously unrecognized cases are identified, a round of surveillance cultures (ie, a point-prevalence survey) in high-risk areas (eg, ICUs or wards where previous cases have been detected) should be considered to identify unrecognized cases. In addition, facilities should ensure a system is in place to promptly notify infection-prevention personnel when carbapenem resistant organisms are identified in the laboratory. All identified carbapenem resistant organisms case-patients should be
placed on contact precautions, and some experts have also recommended patient cohorting and use of dedicated staff for these patients.

3. **Surveillance cultures:** If previously unrecognized carbapenem resistant cases or hospital-onset infections are identified via either clinical cultures or point-prevalence surveys, facilities should consider surveillance cultures from patients with epidemiologic links to carbapenem resistant case-patients. The goal of these cultures is to identify additional unrecognized carbapenem resistant organisms-colonized patients who are a potential source for transmission. The ideal anatomic site to screen for resistant Enterobacteriaceae with surveillance cultures has been investigated in a number of studies. Among these, perianal and rectal cultures are generally the most reliable.

4. **Antimicrobial Stewardship and minimizing devices:** Antimicrobial stewardship has been suggested as an important part of efforts to control MDROs. However, multiple antimicrobial classes have been identified as possible risk factors for infection or colonization with carbapenem resistant organisms. Therefore, antimicrobial stewardship might be most effective if efforts are directed toward an overall decrease in antimicrobial use rather than targeting a specific antimicrobial class. Limiting use of invasive devices is another potentially important intervention for prevention.

5. **Prevention Beyond Acute Care and Role of Public Health:** Although much of the effort for prevention has focused on acute care facilities, nonacute care settings also provide care for patients colonized or infected with these organisms. Limiting prevention efforts to acute care settings fails to take into account the presence of MDROs across different health care settings. Broadening the approach to prevention requires employing setting-specific infection
prevention strategies in all health care arenas but also requires a method for enhanced communication to ensure that proper infection-control practices are continued when patients are transferred between levels of care. [Gupta et al. 2011, Black et al, Flaherty et al, Peleg et al 2008]

3.12. Molecular typing

Identifying different types of organisms within a species is called typing. Understanding pathogen type and its relatedness is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods. The role of pathogen typing is to determine whether epidemiologically related isolates are also genetically related. To determine molecular relatedness of isolates for epidemiologic investigation, technologies based on DNA, are methods of choice. The integration of molecular typing with conventional hospital epidemiologic surveillance has been proven to be cost-effective due to the associated reduction in the number of nosocomial infections. [Singh A et al.2006,]

Historically, this analysis of nosocomial pathogens has relied on a comparison of phenotypic characteristics such as biotypes, serotypes, bacteriophage or bacteriocin types, and antimicrobial susceptibility profiles. This approach has begun to change over the past two decades, with the development and implementation of new technologies based on DNA. These DNA-based molecular methodologies, include pulsed-field gel electrophoresis (PFGE) and other restriction-based methods, plasmid analysis, and PCR-based typing methods such as the random amplified polymorphic DNA (RAPD) assay. The incorporation of molecular methods for typing of nosocomial pathogens assist in obtaining fundamental assessment of strain interrelationship. [Singh et al. 2006, Sabat et al. 2013]
Establishing clonality of pathogens not only aids in the recognition of outbreaks of infection, but also for identification of the source (environmental or personnel) of organisms, distinguish infectious from noninfectious strains, and differentiate relapse from reinfection. Many of the species that are key hospital-acquired causes of infection are also common commensal organisms, and therefore it is important to determine whether the isolate recovered from the patient is a pathogenic strain that caused the infection or a commensal contaminant that likely is not the source of the infection. Likewise, it is important to know whether a second infection in a patient is due to reinfection by a strain distinct from that causing the initial infection or whether the infection is likely a relapse of the original infection. If the infection is due to relapse, this may be an indication that the initial treatment regimen was not effective, and alternative therapy may be required. [Singh et al.2006, Olive et al.1999, ].

The choice of an appropriate molecular typing method depends significantly on the problem to solve and the epidemiological context in which the method is going to be used, as well as the time and geographical scale of its use. All typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance and ease of interpretation.[Tenover et al.1997].

3.12.1. Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) assay, also referred to as arbitrary primed (AP) PCR and DNA amplification fingerprinting (DAF), is a powerful tool for genetic studies. It was first described by Williams et al. (1990) and Welsh & McClelland (1990).

RAPD is based on the parallel amplification of a set of fragments by using short arbitrary sequences as primers (usually 10 bases) that target several unspecified
genomic sequences. Amplification is conducted at a low, non-stringent annealing temperature, which allows the hybridisation of multiple mismatched sequences. When the distance between two primer binding sites on both DNA strands is within the range of 0.1–3 kb, an amplicon can be generated that covers the sequence between these two binding sites. Importantly, the number and the positions of primer binding sites are unique to a particular bacterial strain. RAPD amplicons can be analysed by agarose gel electrophoresis or DNA sequencing depending on the labeling of primers with appropriate fluorescent dyes. Following separation of the amplification products by agarose gel electrophoresis, the pattern of bands obtained is characteristic of the particular bacterial strain. The relationship between strains may be determined by comparing their unique fingerprint information.[Tang et al 1997, Wolska et al 2012]

Although, less discriminatory than PFGE, RAPD has been widely used for the typing of bacterial isolates in cases of outbreaks, because it is simple, inexpensive, rapid and easy in use. The main drawback of the RAPD method is its low intra-laboratory reproducibility since very low annealing temperatures are used. RAPD also lacks inter-laboratory reproducibility since it is sensitive to subtle differences in reagents, protocols, and machines. However, the technique is reliable if the PCR conditions are optimized. It has been reported that the reproducible RAPD patterns are dependent upon the optimal concentrations of DNA in accordance with Taq polymerase and magnesium as well as PCR cycling conditions. Simple, short primers are used without the need of prior knowledge of the template DNA. The selection of an appropriate primer and optimization of PCR conditions are the important factor in RAPD analysis.

RAPD is considered as a simple (PCR amplification with single or two primers → agarose gel electrophoresis → gel staining → interpretation), rapid, highly
discriminating, less costly and simple technique for molecular typing of various microorganisms. RAPD-PCR can be practically applied in most laboratories since it requires no special and/or complex equipment and takes less time and is less laborious. Hence RAPD is useful in the investigation of short term or local outbreaks of disease. It has been successfully applied in the genetic differentiation of Salmonella, E. coli, A. baumannii, P. aeruginosa, Stenotrophomonas maltophilia, Serratia marcescens, Proteus mirabilis, E. cloacae and Burkholderia pseudomallei. [Silbert et al.2004, Singh et al. 2006,Sabat et al.2013].