2.0 REVIEW OF LITERATURE

In the last century, discovery of antimicrobial drugs is undoubtedly one of the most important discoveries. Alexander Fleming in 1928 discovered the first natural antibiotic product β-lactam penicillin. But this drug was not available to 1944. Gerhard Domagk in 1932 synthesized sulfonamide was available in 1938 (Bentley, 2009). Over the next five decades, new classes of antibiotics, both from the synthetic and natural sources were discovered including aminoglycosides, glycopeptides, macrolides, fluoroquinolones, and rifamycins tetracycline, among others.

The introduction of antibiotics reduced the severity of many bacterial infections almost immediately. The success of antibiotic therapy has not only prevent the spread of many diseases, such as bacterial pneumonia and syphilis, it has also led to breakthroughs in surgery and medicine that would not have been possible because of the threatening infection (Taylor, 2011). The benefits of discovery are antibiotic big but-better-often; of course unfortunately, as many things that seem true, it was soon noted that the discovery of antibiotics came with a major problem of bacteria developing resistance mechanisms that can make the strongest antibiotic ineffective.

"When penicillin was discovered, the resistant bacteria were isolated in 1940, it has been found that not only bacteria can be resistant to multiple classes of antibiotics at the same time, but resistance was transferred to sensitive strains" (Davies, 2007). The main reason is the selection of a growing population of bacteria that are now resistant to most antibiotics commonly used. More about the rate of discovery of new antibiotics have declined rapidly over the past three decades to offer so little support to the decreasing effectiveness of first-line therapies currently used.

It is hard to believe that less than 100 years after the introduction of antibiotics, human race is now facing against the possibility of a return to pre-antibiotic age. New strategies to combat these multidrug-resistant bacteria (MDR) are very much required to counter this threat of resistance for the next century and surpasses beyond (Taylor, 2011).

2.1. Antibiotics

2.1.1. Definition of antibiotics.

The term antibiotic refers to a substance produced by microorganisms that inhibits or kills other microorganisms (Harremoes et al., 2001). Now it is widely used and accepted to include synthetic substances that inhibit or kill microorganisms as well as naturally occurring compounds. Antibiotics are classified as bactericidal (cause cell death) like penicillin or bacteriostatic (prevent the growth of bacteria) like chloramphenicol (Walsh, 2010).

"The antibiotics can be classified according to their various mechanisms of action (Table 1) such as (1) interfering with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis, (4) inhibition of metabolic pathways, and (5) disruption of bacterial membrane structure" (Tenover, 2006).

" Mechanism of action	Antimicrobial agent (s)
1. Interference with cell wall synthesis	β- Lactams: penicillins, cephalosporins, carbapenems, monobactams. Glycopeptides: vancomycin, teicoplanin
2. Inhibition of protein synthesis	Macrolides, chloramphenicol, clindamycin, quinopristin-dalfopristin, linezolid Aminoglycosides, tetracyclines
3. Interference with nucleic acid synthesis	Fluoroquinolones, rifampicin
4. Inhibition of a metabolic pathway	Sulfonamides, folic acid analogous
5. Disruption of bacterial membrane structure	Polymixins, Daptomycin" (Tenover, 2006)

Table 1: Antibiotics classification according to their mechanism of action

2.2. Antibiotic resistance

Antibiotic resistance is defined as "the ability of a microorganism to resist antibiotic and survive" (Walsh, 2000). In contrast, the susceptible bacteria would be removed. Over the last seven and half decade the regular use of antibiotics has led bacteria acquiring and showing different resistance mechanisms mediating resistance to one or more drugs: i.e. multidrug resistance (MDR) or where only one or two classes are left, extensive drug resistance (XDR) (Giedraitiene et al., 2011). "Multidrug resistance / XDR has noticed in *P. aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumoniae*, produces Extended spectrum β-lactamas (ESBL), Vancomycin-resistant Enterococci (VRE), Meticillin resistant *Staphylococcus aureus* (MRSA), Staphylococcus *aureus* with vancomycin resistance (VRSA), XDR-*Mycobacterium tuberculosis* (Alekshun & Levy, 2007) *Salmonella* enterica Serovar *typhimurium*, *Shigella dysenteriae*, *Stenotrophomonas* & *Burkholderia*" (Dzidic & Kos, 2008).

Resistance is mainly of two types**-**

2.2.1. Innate resistance

Bacteria can be naturally resistant to an antimicrobial agent. This type of resistance is a result of processes that are adaptive and not necessary, with a given class of antimicrobial agents**.** This intrinsic resistance mechanism refers to the existence of chromosomal resistance genes or mutations in other genes/adjacent DNA altering their expression (Davies & Davies, 2010). For example the natural resistance in Pseudomonas aeruginosa v Sulfonamides, trimethoprim, tetracycline or chloramphenicol, whose low permeability is probably one of the main reasons for its innate resistance. Some other examples include *Stenotrophomonas maltophilia* to imipenem, *Klebsiella* to Ampicillin etc. (Yoneyama & Katsumata, 2006). "Other examples include self-generated antibiotic

resistance, like the outer membrane of Gram-negative bacteria, the absence of a single out for antimicrobial or general absence of target or reaction by antimicrobial" (Wright, 2005).

2.2.2. Adaptive resistance

Adaptive resistance, the main mechanism of antimicrobial resistance, is the consequence of specific evolutionary pressure to develop a self-defense mechanism towards an antimicrobial agent or a class of antimicrobial agents so that bacterial populations previously susceptible to antimicrobial agents are resistant (Toma & Deyno, 2015). Adaptive resistance is given by changes in the bacterial genome. Adaptive resistance in bacteria can be caused by a mutation and this conceded vertically by selection to the daughter cells. The most common is resistance acquired by horizontal transfer of resistance genes between species and strains. Switching of genes are feasible by transformation, transduction or conjugation are the main routes (Rachakonda & Cartee, 2004).

2.3. Carbapenem: Background

This word describes the amount of human hard work that went into the development of this most useful group of antimicrobials and how at every juncture the bacteria have been able to overcome with an appropriate response. In the intervening years since the dawn of the era of antibiotics, drug discovery had become less likely to result from individual efforts.

The 1970s were Olivanic acids, the first natural products (Streptomyces Clavuligerus) identified as a β-lactamase inhibitor. Olivanic acids have a "carbapenem" backbone "and act as wide ranging β -lactamase (Rolinson, 1991). This had poor penetration property and also chemically unstable. Therefore were not further used. "Immediately thereafter, two superior β -lactamase inhibitors were discovered: (i)

clavulanic acid from *S. clavuligerus*, the first clinically available β-lactamase inhibitor and (ii) thienamycin from *Streptomyces cattleya*" (Wallace et al., 2011).

Thienamycin was the first "carbapenem" and would end up as a parent or model connection for all carbapenem. It was notable that thienamycin established strong antibacterial spectrum and β-lactamase inhibitory activity (Wallace et al., 2011). Over time, interest, in this context, grew rapidly, as this showed an inhibitory microbiological action against Gram-negative bacteria, but also thienamycin was unstable in the aqueous phase solution (Kahan, 1979) as stable derivatives was look out for.

"The term "carbapenem" is defined as a fused 4: 5-β-lactam ring blend of penicillins having a double bond between C-2 and C-3, but with the substitution of the carbon for the sulfur in position C-1. The hydroxyethyl side chain is a major feature of carbapenems and is vital for their action" (Zhanel et al., 2005). The broad spectrum of carbapenem activity is linked to its intrinsic resistance to almost all β -lactamases. This β-lactamase permanence is exclusive in comparison with the chains of penicillins and cephalosporins (Zhanel et al., 2005).

Carbapenems are divided into group 1 and group 2. "Group 1 comprised of antibiotics that have narrow spectrum antibacterial activity against non-fermenters Gram-negative bacteria such as ertapenem and Group 2 included antibiotics effective against non-fermenters and recommended to treat hospital infections" (Salabi et al., 2012).

2.3.1. Imipenem

Imipenem (N-formimodoyl derivative of thienamycin) was developed in 1985 (Zhanel et al., 2007) and turn into primary carbapenem obtainable for the curing of complex microbial diseases. Imipenem is effective against most clinically important bacteria including anaerobes. Intrinsically resistant organisms include *Enterococcus*

faecium, methicillin resistant *Staphylococcus aureus* (MRSA), coagulase negative *staphylococci, Chlamydia trachomatis and Stenotrophomonas maltophilia*.

Most β-lactamases is unable to destroy this including those that mediate resistance to cefuroxime, cefotaxime and ceftazidime (ESBL). *Pseudomonas aeruginosa* can become resistant during therapy due to a mutation which reduces bacterial permeability to the drug. Cross-resistance to other β-lactams is unusual (Bowlware & Stull, 2004). Imipenem is rapidly degraded by dehydropeptidase (DHP-1) produced by the human kidney and has an adverse toxic effect on the kidney, therefore imipenem should be given with cilastatin in the ratio of 1:1 to act as an inhibitor of the dehydropeptidase enzyme and to neutralize the toxic effect of the imipenem (Rodloff et al., 2006).

"Besides imipenem, later identified, a closely related carbapenem namely panipenem is too subject to reduction of DHP-1 in the brush border of renal tubules and requires co-administration of an inhibitor" (Goa and Noble, 2003). In this case, called an organic anion tubular transport inhibitor, Betamiprion was used to avoid inclusion in the renal tubules (Goa and Noble, 2003).

So carbapenem with better permanence and with a wider spectrum for example ertapenem, meropenem, doripenem and tebipenem developed.

2.3.2. Ertapenem

"Ertapenem is structurally analogous to meropenem, though with a meta-substituted benzoic acid group at position 2" (Livermore et al., 2003). The meta replacement increases the molecular credence and lipophilicity of the molecule, and creates a negative overall on the benzene ring, at physiological pH. The ionization of the bond and the longer half-life of ertapenem is comparative to additional carbapenems (Zhanel et al., 2005). It has unique antimicrobial spectrum because of larger and more negatively charged

is likely to permeate Gram-negative cell walls more slowly than meropenem (Livermore et al., 2003).

Ertapenem is premeditated to be effective against gram-negative bacteria. It is not lively against methicillin-resistant Staphylococcus aureus (MRSA), resistant to ampicillin Enterococci, Pseudomonas aeruginosa or Acinetobacter species. Ertapenem also has action clinically valuable against anaerobic bacteria. Ertapenem is used as first-line treatment for community-related infections. It is not used as an pragmatic treatment for the nosocomial infections because of their lack of activity against Pseudomonas aeruginosa. In the common practice, it is held principally for use against broad spectrum β–lactamases (ESBL) producing AmpC Gram-negative bacteria (Livermore et al., 2001).

2.3.3. Meropenem

Meropenem differ from imipenem by a pyrrolidinyl substituent on 2 Position. This is the major basis for its superior action against Gram-negative organisms together with Pseudomonas aeruginosa in contrast to imipenem (Zhanel et al., 1998).

Meropenem is a broad-spectrum antibiotic to treat a variety of infections such as meningitis and pneumonia. "It penetrates fine into many tissues and body fluids such as cerebrospinal fluid, bile, heart valves, lungs, and peritoneal fluids" (Rao et al., 2012).

Meropenem is bactericidal except for Listeria monocytogenes where it is bacteriostatic. It inhibits the synthesis of the bacterial wall as other β-lactam antibiotics, but on the contrary other β -lactamases, which is extremely resistant to deprivation by β-lactamases or cephalosporinases. Contrasting to imipenem, meropenem is stable against dehydropeptidase-1 and therefore can be given without cilastin (https://en.wikipedia.org/wiki/Meropenem).

2.3.4. Doripenem

Doripenem was allowed for the use by the FDA in 2007. Presence of sulphamoylaminoethyl pyrrolidinylthio group in its side chain at position 2, resulted in its increased action towards non-fermentative Gram-negative bacilli (Patterson & Depestel, 2009).

Doripenem is an broad-spectrum antibiotic. It forms a stable acyl enzymes and causing weakening of bacterial cell wall and consequently lead to cell wall rupture as a result of osmotic pressure forces (Stratton, 2005). It has greater in vitro potency than meropenem against *Pseudomonas aeruginosa* while its activity against extended spectrum β-lactamases (ESBL) producing Gram-negative organisms is similar to that of meropenem.

2.3.5. Tebipenem: "pivoxil, a new oral carbapenem, has a 1-(1,3-thiazolin-2yl) azetidin-3-ylithio group at the 2 position. Tebipenem is highly stable to DHP-1 and is converted by an esterase in the intestine into its active metabolite, which is then absorbed into the bloodstream" (Kobayashi et al., 2005).

2.4. Mode of action

All beta-lactam antimicrobial agents, including carbapenem show bactericidal activity binding to penicillin binding proteins (PBPs) (Norrby, 1995). "The binding of beta-lactam molecule to PBPs prevents bacteria from transpeptidation (cross-linking) of peptidoglycan strands thereby preventing the synthesis of an intact bacterial cell" (Zhanel et al., 2007).

Imipenem bind firstly to PBP2 then to PBP 1a and 1b and has weak affinity for PBP-3 (Livermore et al., 2003). Meropenem & ertapenem bind more strongly to PBP2 followed by PBP-3, but also has strong affinities for PBP 1a and PBP1b (Livermore et al., 2003). It has been reported that doripenem has a strong affinity for PBP targets, which are

specific species, PBPs 1, 2 and 4 in Staphylococcus aureus; PBP2 in the Escherichia coli and Pseudomonas aeruginosa PBP-3, (Jones et al., 2004). The affinity of biapenem is stronger for PBPs 1 and 3 in Staphylococcus aureus PBPs 1a, 2 and 4, in Escherichia coli and PBPs 1a 1b in Pseudomonas aeruginosa (Yang et al., 1995). Tebipenem showed a strong affinity for PBP 1a, 2b and 3 in isolates of Streptococcus pneumoniae (Kobayashi et al., 2005).

In Gram-negative bacteria, carbapenems achieve the rapid bactericidal effect by binding with the highest affinity for PBP 1a, 1b and 2, instead of PBP-3, which is the primary aim of aminopenicillins and cephalosporins (Norrby, 1995). Carbapenem achieve lysis of cell without filamentation, as seen with agents acting primarily on PBP-3 as the third generation cephalosporins. This result in support of a smaller increase of mass of bacteria cells before lysis and less release of endotoxin (Jackson & Kropp, 1992).

2.5. Mode of resistance

The key mechanisms of resistance to carbapenems comprise the production of β-Lactamases, efflux pumps and mutations that amend the expression and / or purpose of porins and penicillin binding proteins. "The combinations of these different mechanisms can lead to elevated to very high resistance to carbapenems in certain types of bacteria, such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*" (Rodriguez- Martinez et al., 2009).

There is a difference between resistance to carbapenems in Gram-positive cocci and Gram-negative rods. "In Gram-positive cocci, carbapenem resistance is characteristically as a result of the substitutions on amino acid sequences of PBPs or acquisition of production a novel carbapenem-resistant penicillin binding protein" (Koga et al., 2009, Matsumoto et al.2007) with expression of β-lactamases & efflux pumps, plus

porin loss & changes in penicillin binding protein, all of which are coupled with carbapenem resistance in Gram-negative bacteria (Nordmann et al., 2011 d).

2.6. Carbapenem hydrolyzing enzymes

Carbapenem hydrolyzing enzymes i.e. carbapenemases, the most important cause of carbapenem resistance, are specific β-lactamases that have the ability to hydrolyze carbapenems. "Production of carbapenemases appears to be the major cause of carbapenem resistance given that the documentation of its distribution in different types of bacteria including fermenters and non-fermenters is widespread" (Walsh, 2010). These periplasmic enzymes hydrolyzed carbapenems & thus preventing the drug from reaching the target PBP. So far, these periplasmic enzymes were mainly specific for some types and encoded on the chromosome. Since plasmid carbapenemases appeared in the 1990s, they observed in different organisms.

For β-lactamases two important classifications are used: the Ambler classification (molecular) and the Bush-Jacoby classification (Functional) (Ambler, 1980; Bush, 1988) and "two major molecular families: metallo-carbapenemases and serine carbapenemases which are differentiated by their hydrolytic mechanism at the active site" (Bush et al., 1995).

Metallo-enzymes found to be ethylenediaminetetraacetic acid (EDTA) inhibited, and have at least one zinc atom in the active site, which assists in the hydrolysis of β-lactam ring (Frere et al., 2005). In the 1980's, serine enzyme become known and were determined to be clavulanic acid and tazobactam inhibited, excluding EDTA (Medeiros & Hare, 1986 Yang et al., 1990; Rasmussen & Bush, 1997)

2.6.1. **Functional** or **Bush-Jacoby classification**

This functional classification of the β-lactamase enzyme involved the determination of substrate; enzyme kinetic inhibition profiles of hydrolysis; the biochemical analysis of the enzyme and the determination of the isoelectric point (Sykes & Mateus, 1976). This functional Classification of Karen Bush and others have been many through many reviews and now share β-Lactamases in four functional groups (1-4). Group 2 has a plurality of subgroups, which are differentiated by specific substrate or group inhibitor profile. In this functional classification schemes, the carbapenemases are placed under group 2f and three (Bush et al., 1995).

2.6.2. Molecular or Ambler classification

"Molecular classification classified β-lactamases in accordance with the amino acid sequences in four groups (A-D)" (Bush & Jacoby, 2010). Although this classification correlates well with the functional scheme but lacking the details of the β-lactamase enzyme activity. The molecular classes A and D contain β-lactamases with serine while Group B metallo-beta-lactamase contains zinc at its active sites. "Group B enzymes require one or two Zn cations for the activity and are based on sequence alignments and structural analysis divided into three groups B1, B2 and B3. Subclasses B1 and B3 have two zinc ions, while the class B2 has only one Zinc ions" (Hall & Barlow, 2005). B2 enzymes are mainly carbapenemases while enzymes B1 and B3 are broad hydrolysis spectrum (Frere et al., 2005).

2.6.3. Class A- Serine carbapenemases

Class A (serine carbapenemases) at 2f functional group has emerged sporadic in clinical isolates since their first discovery more than 20 years ago (Medeiros & Hare, 1986). "These carbapenemases have been detected in *Serratia marcescens*, *Enterobacter*

cloacae, and *Klebsiella* species as single isolates or in small outbreaks" (Nordmann et al., 1993). Bacteria expressing this carbapenemases are shown by a decreased sensitivity to imipenem, however MICs may range from slightly elevated (eg, imipenem MIC of ≥ 4μg / ml) to totally resistant. These β-lactamases may thus remain undetected after routine susceptibility tests.

"There are three major families of class A serine carbapenemases namely KPC NMC/IMI and SME enzymes. All these enzymes have the ability to hydrolyze a broad variety of β-lactams, including carbapenems; cephalosporins, penicillins and aztreonam, and all are inhibited by clavulanate and tazobactam, placing them in the group 2f functional subgroup of β-lactamases. Another member of this class is the GES β-lactamases, was originally identified as an ESBL family, but over time variants were discovered that had low, but measurable, imipenem hydrolysis" (Nordmann et al., 2012 b). This subgroup of GES enzymes is also classified as functional group 2f carbapenemases.

All enzymes have the capacity to hydrolyze penicillins & extended spectrum cephalosporins, aztreonam & carbapenems. Clavulanic acid and tazobactam inhibited these enzyme excluding EDTA. These enzymes are in the functional subgroup 2f. "Since most of the class A carbapenemases are chromosomal (EMS, IMI, NMC), however plasmid-mediated carbapenemases (KPC, GES) are on the increase" (Queenan & Bush, 2007).

2.6.3.1. Chromosomally encoded class A enzymes

a) SME (*Serratia marcescens* enzyme)

S. marcescens isolated from England in 1982. There have been only 3 types of SME (SME-1, SME-2 and SME-3). These enzymes were sporadic and observed in S. marcescens strains all over the United States. No clonal dissemination were observed among these isolates (Nordmann et al., 2012 a).

b) IMI (Imipenem hydrolyzing β-lactamase):

IMI-1 enzyme was first observed in an isolate of *Enterobacter cloacae* in the United States in 1984. Since then, this has been rarely observed in clinical isolates of E. cloacae in United States, France and Argentina. Consequently IMI-2 was reported from China that was plasmid encoded. "This chromosomal β-lactamases can be induced in response of imipenem and cefoxitin" (Yu et al., 2006).

c) NMC (Non-metallo carbapenemase):

"NMC-enzyme was isolated from *Enterobacter cloacae* isolated in France during 1990 and later reported from Argentina and the United States. NMC-A and IMI have 97% amino acid homology, and are related to SME-1, which has 70% amino acid homology" (Radice et al., 2004). "Although these enzymes have a large hydrolysis spectrum, inlcuded cephalosporins, aztreonam and carbapenems, cefoxitin and extended spectrum cephalosporins were ineffectively hydrolyzed" (Radice et al., 2004).

2.6.3.2. Plasmid encoded class A carbapenemases:

a) KPC (Klebsiella pneumoniae carbapenemase)

The KPC-like carbapenemases known as *Klebsiella pneumoniae* carbapenemases. They are the most abundant of molecular class A and functional group 2f. "KPC may hydrolyze various different antimicrobial agents, for example fluoroquinolones, aminoglycosides, and ß-lactams, with carbapenems, cephalosporins, penicillins and aztreonam" and are inhibited by clavulanic acid and tazobactam" (Endimiani et al 2009; Castanheira et al., 2008; Queenan and Bush, 2007).

"KPC enzyme was first reported in a Klebsiella pneumoniae isolate from United States in1996 and then reaches in New York" (Yigit et al., 2001, Patel et al., 2009, Kitchel et al., 2009). The gene of interest was related with a large plasmid. KPC are found largely in

Klebsiella pneumoniae; conversely, they were also found in several others Enterobacteriaceae, with *Escherichia coli* (Urban et al., 2008), *Enterobacter* spp, (Bratu et al., 2005), *Salmonella* species (Miriagou et al., 2003), *Proteus mirabilis* (Tibbetts et al., 2008) and *Citrobacter freundii* (Rasheed et al., 2008) and other non Enterobacteriaceae, with *Pseudomonas aeruginosa* (Villegas et al., 2007) and more of late in a strain of *Acinetobacter baumannii* (Robledo et al., 2010).

In contrast to the penicillin & cephalosporin, the hydrolysis rates of imipenem, meropenem, cefotaxime and aztreonam are less than ten times. Irrespective of the ability of these enzymes to hydrolyze carbapenem, in many cases, the resistance is not evident because the MIC did not match the cut off point. This has led to under recognition of a number of KPC producers (Queenan & Bush, 2007).

"There are two characteristics that separate KPC from other enzymes of functional group 2f. First, KPC enzymes are found in transmissible plasmids; second, the hydrolysis spectrum of the substrate comprises aminothiazolexim cephalosporins, such as cefotaxime" (Bratu et al., 2005).

Sixteen different variants (KPC-2 to KPC-17) in the KPC family have been reported, and KPC-2 and KPC-3 are the spotlight of current studies. The KPC-16 variant isolated from *Klebsiella pneumoniae* strain in a Chinese hospital was a newly discovered KPC enzyme. To compare the properties of KPC-15 and KPC-2, variants were determined for susceptibility testing, PCR amplification and which is then followed by sequencing and plus by the research on the kinetic parameters.

The isolate haven the KPC-15 exhibited resistance to conventional antimicrobial, in particular, carbapenem antibiotics, and the strain haven KPC-2 showed resistance to carbapenem antibiotics, but both strains were susceptible to polymyxin B and colistin (Wang et al., 2014).

"Infections produced by KPC are common among hospital pathogens and related with treatment failure and with high mortality rates reaching at least 50%" (Landman et al., 2007 Gasink et al., 2009). Combination therapy is suggested as a action for KPC infections (Lee and Burgess, 2012). The multiplex real-time PCR assay with molecular beacons has been found to be robust, susceptible and definite, allowing a high yield detection and categorization of all KPC variants (Chen et al., 2011).

"In addition to their broad-spectrum hydrolytic capabilities that include all classes of β-lactams, including monobactams, the KPC enzymes are never found as a single enzyme in a producing isolate" (Bush, 2013). In enteric bacteria, KPC-2 and KPC-3 enzymes are often found with the TEM-1 penicillinase. While many researchers did not account the presence of chromosomal β-lactamases in KPC producing enteric bacteria, it is possible that most Enterobacteriaceae also producing additional group 1 or group 2e cephalosporinases in the blend of β-lactamases, except Escherichia coli & Klebsiella (Bush, 2013).

"Among KPC producing organisms, aminoglycosides and fluoroquinolones resistance determinants have been frequently reported, with a variety of aminoglycosides-modifying enzymes affecting various aminoglycosides and *qnr* genes conferring decreased susceptibility to many fluoroquinolones" (Bush, 2013). Acetyltransferase genes are among the most prominent determinants of aminoglycoside resistance, among rmt methyltransferases noted in many strains. In addition, genes related to trimethoprim or sulphonamide resistance such as *sul* and *dfr* genes have been observed. Less frequent are *cat* genes encoding chloramphenicol acetyltransferases, *arr* genes encoding ADP-ribosylation of rifampicin, or the *tet* (G) tetracycline efflux pump (Bush, 2013).

b) GES (Guiana extended spectrum):

"This enzyme was first observed in a *Klebsiella pneumoniae* isolate from French Guiana in 2000. The enzymes of the GES family differ from each other by 1-4 amino acid substitutions. The genes encoding GES family of enzymes are located in integrons on plasmid" (Poirel et al., 2007). There are 22 known GES types. "These have been observed in *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa* isolates from different nations (Greece, France, Portugal, South Africa, French Guiana, Argentina, Japan and Korea)" (Ciobotaro et al., 2011). In an Escherichia coli isolate the gene GES (blaGES-7) is chromosomally localized and is the only blaGES gene not identified in an integron.

"The GES-14 variant is one of these GES-type carbapenemases and has been identified in *Acinetobacter baumannii* in France in 2011" (Bonnin et al., 2011). "The foremost GES type carbapenemase was identified from a *Pseudomonas aeruginosa* isolate was GES-2, contary from GES-1 by a single amino acid substitution" (Poirel et al., 2001). "The GES-5 variant, which has a significant carbapenemase activity has also been reported in *Pseudomonas aeruginosa* isolated from China" (Wang et al., 2006), South Africa (Girlich et al. 2012 b) and Turkey (Malkocoglu et al., 2017). These blaGES-type genes are part of class 1 integron structures (Labuschagne et al., 2008). Lately a new variant of GES, GES-18, known from a *Pseudomonas aeruginosa* isolated from Belgium. It was different from GES-5 by a substitution of amino acids and it hydrolyzed carbapenems also. (Bebrone et al., 2013).

2.6.3.3. Class B carbapenemases (metallo-β-lactamases)

The metallo-β-lactamases (MBLs) are the most molecularly diverse carbapenemases and are the biggest clinical threat. "The active site often contains two zinc

ions, which coordinate and present polarized water ions for the oxyanion attack on the β-lactam ring" (Ganta et al., 2009).

Among the β-lactamases, attack and hydrolysis is exclusive and is important from clinical point, as MBL does not form a stable or pseudo-stable intermediary i.e. does not actually bound to the β-lactam substrate and thus escape the effect of β-lactamase inhibitors for example clavulanic acid and sulbactam (Walsh et al., 2005).

MBL is capable of hydrolyzing all penicillins, cephalosporins, β-lactamase inhibitors and also carbapenems but carbapenems are sensitive to inhibition by aztreonam and chelating metal ions (EDTA) (Frere et al., 2005). However, many clinical isolates are now presenting with an array of β-lactamases such that pan-β-lactamase resistance is a common phenomenon in some countries like India and Greece (Walsh, 2010).

The majority of MBL genes such as IMP, VIM were found as gene cassettes of class 1 integrons; a small number of IMP genes are located in Class 3 integrons. "Genetic analysis of the regions around SPM 1 gene showed that it was not part of integrons, but was instead connected to the common regions that have a new type of transposable structure with impending recombinase & promoter. The machinery of carbapenem hydrolysis is complex and ranges from one to MBL to others" (Walsh et al., 2005).

MBL was never classified as a single β-lactamase in an organism. Regardless of MBL, that enzymes most often associated enzyme is TEM-1 next SHV or CTX β-Lactamases. As described over with the KPC carbapenemases were SHV-11, SHV-12 and CTX-M-15 are often identified in IMP and NDM-1 producing strain with Spanish VIM producing strains reported as CTXM-3 or 9 ESBL. In contrast to other carbapenemase producing isolates, strains with NDM-1 have been reported to produce a variety of CMY cephalosporinases (Bush, 2013).

Chromosome borne metallo-β-lactamases:

"The first MBL were found in environmental and opportunistic pathogenic bacteria *(Bacillus cereus* (BCII), *Aeromonas spp.* (CphA) and *Stenotrophomonas maltophilia* (L1) as chromosomally encoded enzymes. These bacteria also produced additional serine β-lactamases and the both enzymes were inducible by exposure to β-lactams. Chromosomal MBL was also found in few strains of *Bacteroides fragilis* (CcrA). Fortunately, with the exception of *Stenotrophomonas maltophilia*, these bacteria have not been frequently associated with serious nosocomial infections, as they are generally opportunistic pathogens, and the chromosomal metallo-β-lactamase genes are also not easily transferred" (Queenan and Bush, 2007).

Phylogenetic investigation explains the subsistence of three MBL lineages: B1, B2 and B3. These three subclasses are separated based on hydrolysis affinities, zinc affinities for two binding sites and a combination of structural characteristics. Subclasses B1 and B3, classify on the basis of amino acid homology, combine two zinc atoms used for optimal hydrolysis whereas in enzymes B2 subclass are repressed when a second zinc is bound (Queenan and Bush, 2007).

"Subgroup B1 includes the acquired enzymes of the VIM, IMP, GIM, SPM, SIM, AIM, DIM, and NDM types. Several variants of the VIM, IMP, and NDM types have been found in *Klebsiella pneumoniae* and other Enterobacteriaceae like *Escherichia coli, Enterobacter cloacae* (mainly VIM and IMP)*, Serratia marcescens* (mainly IMP)*, and Proteus mirabilis* (mainly VIM)" (Tzouvelekis et al., 2012). *Bacillus cereus* (BcII) are also the well characterized MBLs which was the first to be discovered, and *Bacteroides fragilis* (CcrA) Subclass B1 enzymes are encoded by mobile genetic elements, posing the greatest threat of all the MBLs. Subclasses B1 and B3 have broad spectrum substrate profile that include penicillins, cephalosporins and carbapenems (Horsfall et al., 2011). "Subclass B3

includes the tetrameric enzyme L1 from the opportunistic pathogen *Stenotrophomonas maltophilia* GOB-1 from *Chryseobacterium meningosepticum*, THIN-B from *Janthinobacterium lividum*, FEZ-1 from *Fluoribacter gormanii* and Mbl1b from *Caulobacter crescentus* (Horsfall et al., 2011). Subclass B2 has a narrow substrate spectrum limited to carbapenems. It contains the very similar *Aeromonas* chromosomal enzymes, CphA and ImiS" (Horsfall et al., 2011).

Plasmid borne metallo-β-lactamases:

Plasmid-mediated metallo-beta-lactamases (MBL) now have an important meaning because of its circulation around the world. Most mobile MBL genes are found as gene cassette. These include bla_{IMP}, bla_{VIM}, bla_{GIM}, and bla_{SIM} (Carattoli, 2010). "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 metallo β-lactamase-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) (Cornaglia et al., 2011).

I MP (Active on imipenem):

A transferable carbapenem resistance was first noted in a *Pseudomonas aeruginosa* strain isolated from Japan in 1990 (IMP-1). They were reported later in four *Serratia marcescens* strain isolated in Japan. For years, it was believed that bacteria producing IMP type MBL were restricted to Japan. This belief was altered with the recognition of IMP and IMP 2-5 in *Acinetobacter baumannii* in Italy (Riccio et al., 2000) and Portugal (Da Silva et al., 2002).

Among the 51 known IMP variants, 32 have been reported from *Pseudomonas aeruginosa* and have been identified throughout the world (Potron et al., 2015). These

enzymes were also reported from Enterobacteriaceae, but more prevalent in *P. aeruginosa* & *A. baumannii* (Bedenic et al., 2014). "These enzyme hydrolyzed imipenem, penicillins and broad spectrum cephalosporins, excluding aztreonam. Hydrolytic activity was inhibited by ethylenediaminetetraacetic acid (EDTA) and reinstate by the addition of Zn2" (Mathers et al., 2009).

VIM (Verona integron mediated metallo-β-lactamase)

The second dominant group acquired MBL is VIM subclass, also known as European MBL, due to the prevalence of this enzyme in continental countries (Walsh et al., 2005). Currently, known 18 variants identified in different micro-organisms and reported in several countries around the world (Bush & Jacoby, 2010). VIM-1 variant was the first to be identified in *Pseudomonas aeruginosa* isolated from wound of an ICU patient at the University Hospital of Verona in Italy in 1997 (Lauretti et al., 1999). Shortly thereafter outburst of *Pseudomonas aeruginosa* resistant to carbapenems and VIM-1 producer, at the same organization were reported (Cornaglia et al., 2000).

They were suspected of producing MBL and this suspicion was consigned on 8 isolates possessed the blaVIM-1 gene. The succeeding variant of this VIM-2 subclass was noted in France in *Pseudomonas aeruginosa* isolate recovered from blood culture of a patient with leukemia (Poirel et al., 2004 c) VIM has been reported in various geographical regions. Graphics in different bacterial species being responsible for outbreaks like the one that occurred in a Greek hospital (Mavroidi et al., 2000).

Notably, enzymes of the VIM type that have been generally identified in Enterobacteriaceae have not often recognized in *Acinetobacter baumannii*. There are few reports of VIM-1(Greece) VIM-2 (South Korea) VIM-4(Italy) VIM- 6 (India) and VIM-11(Taiwan) (Potron et al., 2015).

"Although VIM enzymes share <40% amino acid identity with the IMP-type enzymes, they share the same hydrolytic spectrum" (Nordmann & Poirel, 2002). "VIM-1 was the first MBL identified in *Pseudomonas aeruginosa* and has been reported from several European countries" (Lauretti et al., 1999). However, VIM-2 is now the most widespread MBL in *Pseudomonas aeruginosa* as a source of multiple outbreaks (Walsh et al., 2005). Twenty-three of the forty-six VIM variants have been identified in *Pseudomonas aeruginosa.*

"IMP and VIM producing organisms also exhibit high resistance to aminoglycosides, again due to aminoglycosides-modifying enzymes such as acetyltransferases encoded by genes such as aacA4, aminoglycoside adenylyltransferase encoded by genes such as aadA1, or phosphotransferases encoded by genes such as aphA6" (Ramirez & Tolmasky, 2010).

A larger range of resistant factors has been reported from clinical strains having VIM & IMP carbapenemase than KPC, mainly because the former were longer in these isolates as compared to KPC. This variety may also be due to their more frequent occurrence in pseudomonal isolates, which tend to accumulate large numbers of resistance factors. The acquired resistance to chloramphenicol, trimethoprim and fluoroquinolones are common, besides the MBL production and also the production of its companion's β-lactamases (Bush, 2013).

SPM-1 (Sao Paulo metallo-β-lactamase)

The third subclass MBL acquired was described in *Pseudomonas aeruginosa* obtained from urine culture of a patient with acute lymphoblastic leukaemia hospitalized in the Hospital Complex São Paulo, Brazil in 2001. After five days, an isolated *P. aeruginosa* with the same susceptibility profile to antimicrobials was obtained

from blood culture (Toleman et al., 2002). This MBL was named SPM-1 and hydrolyze all β-lactam antibiotics, preferentially cephalosporins, being unable to hydrolyze aztreonam, ticarcillin and clavulanic acid (Murphy et al., 2003).

"Genetic investigation of regions covering the entire SPM-1 gene showed that it is not part of a integron but was associated with common regions that have a new kind of transposable composition with impending promoter and recombinase sequences" (Poirel et al., 2004). To date, SPM-1 was only found in *Pseudomonas aeruginosa* and this enzyme appears to be restricted to Brazil, because there is no re chelates producing strains MBL SPM-1 type in or other countries (Zavascki et al., 2005).

GIM (German imipenemase)

In 2002, five isolates of multidrug resistant *Pseudomonas aeruginosa* were obtained from different patients Dusseldorf, Germany. These isolates were from respiratory tract and were resistant to all β-lactam antibiotics, aminoglycosides and quinolones, being sensitive only to polymyxin B. Many phenotypic tests indicated the production and analysis MBL genotypic revealed a new gene called MBL bla_{GIM-1} (Castanheira et al., 2004).

Representing the fourth subclass acquired MBL; GIM-1 has a broad profile hydrolysis, but has no specific substrate, and also does not hydrolyze aztreonam and β-lactamase inhibitors. As most of the genes encoding the MBL, GIM-1 is found in class 1 integron, which, genes encoding resistance to aminoglycosides and a gene for blaOXA-2 (Castanheira et al., 2004).

SIM (Seoul Imipenemase)

SIM-1 was the fifth subclass MBL to be identified. This new MBL found in isolates of imipenem-resistant *Acinetobacter baumannii* and *Pseudomonas* species

obtained from patients with pneumonia infection urinary tract infections, at a hospital in Seoul, Korea, in the period between 2003-2004 (Lee et al., 2005). These isolates had low MICs for imipenem and meropenem (8 to 16 mcg / ml), but had a phenotype of multiple drug resistance. These isolates were sensitive to ciprofloxacin, but resistant or intermediary to other antimicrobials, including broad - spectrum cephalosporins, ampicillin, sulbactam and all tested aminoglycosides. The gene encodes the SIM-1 is located in a integron of class 1. "The enzyme SIM-1 (for "Seoul imipenemase") is more related to IMP type enzymes and has the closest amino acid identity to the IMP family (64 to 69%)" (Lee et al., 2005).

NDM (New-Delhi metallo-β-lactamase)

"It refers to New-Delhi metallo β-lactamase belong to the Ambler Molecular class B and group 3 according to Bush-Jacoby functional classification. It was first identified in 2008, from *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from a patient that acquired an infection in New Delhi, India" (Kumaraswamy et al., 2010). This is a broad spectrum β-lactamase, which inactivates all β-lactams, excluding aztreonam.

"The increase in the number of NDM-positive bacteria has a complex mechanism that comprises diverse species such as *Klebsiella pneumoniae* and *Escherichia coli* and inter-strain, inter-species spread of dissimilar plasmid with blaNDM-1 gene in more than 40 countries of the world" (Fallah et al., 2011). Indeed the spreading of genes erstwhile dominant spreading means (Johnson & Woodford, 2013). Many of the earliest NDM isolates showed a link to the Asian subcontinent mainly India (Kumaraswamy et al., 2010). In the United Kingdom, NDM-1 has been known from the 2008, with all an epidemiological connection to India (North et al., 2011 c). "Additionally, reports from various countries around the world (Remote countries and the Middle East, USA and

Canada and many in Europe) have recognized NDM-1 presence with connection to India" (Fallah et al., 2011). On the other hand, these links were not always connected with hospitalization because some isolates were obtained from the community infections in patients who only had a trip to India.

Additionally, as many reports described the episode of NDM-1 positive bacteria in Indian hospitals (Johnson & Woodford, 2013), a measure of occurrence of the NDM-1 gene in drinking water and seepage samples in New Delhi found that it was carried by various strains in a worrisome frequency (Walsh et al., 2011). This indicates that the NDM-proliferation has crossed the hospital walls and that environmental transmission is taking place (McKenna, 2013). Additionally, many patients colonized with NDM-1 producers originated from the Balkan states and showed it as a second reservoir (Livermore et al., 2011 b).

In addition, many reports have noted the presence of isolates from many countries with no travelling history to India, indicated the risk of spread by asymptomatic patients and narrow spread subsequent introduction (Cendejas et al., 2010). "NDM-1gene is passed by different plasmids are also linked with several resistance genes with carbapenemases, cephalosporinases, macrolide, rifampicin and sulfamethoxazole resistance, a blend that makes these isolates resistant to many drugs" (Walsh et al., 2011). "NDM-1 showed a broad substrate profile that can hydrolyze penicillins, cephalosporins and carbapenems" (lower activity compared to cefoxitin and ceftazidime) (Yong et al., 2009).

The previously isolated NDM variants are not as much diversified than VIM and IMP variants. There are 16 variants for the NDM that were identified (NCBI, 2016). The very first one NDM known after NDM-1 is NDM-2, which vary from a single amino acid replacement (Pro to Ala) at position 28. This variant was not identified in Enterobacteriaceae. NDM-3 has amino acid substitution at position 95 (Asp to Asn).

“Interestingly NDM-4, NDM-5, NDM-7, NDM-8 and NDM-12 have change in peptide sequence at position 154 (Met to Leu present the M154L mutation in Loop L8 (position 150a according to BBL numbering)” (Nordmann et al., 2012 a). “The biochemical characterization of NDM-4 showed a little increase in activity towards cephalosporins (ceftazidime, cephalothin and cefotaxime) and carbapenems (imipenem and meropenem) associated with small increments in minimal inhibitory concentration (MIC) for carbapenems” (Nordmann et al., 2012). “MIC trials with NDM-5 presented high levels compared to NDM-1 for ceftazidime, cefotaxime and cefpirome (Hornsey et al., 2011). There was also an increase in carbapenem MICs, but only if the natural promoter was used for expression in Escherichia coli” (Meini et al., 2014).

As 40 and 44 sequences were isolated over a period of 15 or 26 years for IMP and VIM, and with the rapid increase of NDM gene, it would be expected that more NDM variants are to be discovered in the coming few years.

“Recently, a novel MBL named FIM-1, exhibiting its highest similarity (40% amino acid identity) with NDM-type enzymes, was reported in a *Pseudomonas aeruginosa* isolate from Italy. The blaFIM-1 gene was chromosomally located and was associated with ISCR19-like elements that were likely involved in its capture and mobilization” (Pollini et al., 2013) but its origin remains unknown.

In addition to the above features, aminoglycoside resistance mechanism has become an expected companion to this NDM-1. Apart from the aminoglycosidase modifying enzymes other important factors responsible for the resistance includes 16srRNA methylase encoding genes armA, rmtB and rmtC. These methylase functions are particularly upsetting in terms of novel antibiotic development (Bush, 2013). ACHN-490, a latest aminoglycoside, which elude the majority of plasmid-encoded aminoglycosidase-modifying enzymes are unluckily affected 16S rRNA methylation, hence depicting the

antibiotic fruitless alongside the organism encoding NDM-β-lactamases (Livermore et al., 2011 a).

ACHN-490, a new aminoglycoside that evades most plasmid encoded aminoglycosidase modifying enzymes is unfortunately affected 16srRNA methylation thus rendering the antibiotic ineffective against organism encoding NDM β-lactamases (Livermore et al., 2011 a).

2.6.3.4. Class D carbapenemase

"Class D β-lactamases are also known as oxacillinase or OXA type β-lactamases (OXAs) because of their ability to hydrolyze oxacillin" (Poirel et al., 2010 b). "OXA-48, belongs to the Ambler Molecular Class D (Ambler, 1980), and group 2d according of Bush-Jacoby Classification" (Bush et al., 1995).

During the late 1970s and early on 1980s OXA was characterized as one of the most widespread plasmid-encoded β-lactamase family (Matthew, 1979, Medeiros, 1984, Simpson et al. 1980). This hydrolyzed imipenem and meropenem feebly and do not hydrolyzed extended spectrum cephalosporins and aztreonam. These enzymes further also hydrolyzed cloxacillin, cephalosporins and some carbapenems. These enzymes are normally not affected by β-lactamase inhibitors but may be inhibited in laboratory environment by sodium chloride (Poirel & Nordmann 2006). "Their activity is inhibited by clavulanic acid, an uncommon property for oxacillinases, except for OXA-23, which is resistant to clavulanic acid. OXA-24 and OXA-27 hydrolyzed benzylpenicillin and cephaloridine, while oxacillin and cloxacillin hydrolysis is not detectable" (Afzal-Shah et al., 2001).

Although there are increasing reports of oxacillinases in Enterobacteriaceae especially in *Klebsiella pneumoniae*, OXA carbapenemase activity are often found in

Acinetobacter species, and in particular possessing class D carbapenemases. The foremost OXA B-lactamase as carbapenemase activity was purified from a multi-drug resistant strain of *Acinetobacter baumannii* as IRA-1 (for "Acinetobacter resistant to Imipenem ") was noted in 1993. (Paton et al., 1993). This AR-1 enzyme was soon confirmed that it resides on a large plasmid (Scaife et al., 1995). "Sequencing of AR-1 revealed that it belongs to class D OXA family of β-lactamases and the enzyme and afterward altered to OXA-23" (Donald et al., 2000).

The OXA group showing carbapenemase activity is divided to five subfamilies namely OXA-23, OXA 24/OXA40, OXA-48/181, OXA-58 and OXA-51. The first four groups are present on the transmissible plasmids, while the last group, OXA-51, are chromosomally located and intrinsic in *Acinetobacter baumannii* species. OXA48/181 is specific to Enterobacteriaceae and is a growing problem in North Africa, Turkey and Greece (Evans & Amyes, 2014).

"Presently, there are nine major subgroups of OXA Carbapenemases based on amino acid homology and are divided into nine main subgroups (Walther Rasmussen & Hoiby, 2006). Subgroups 1, 2 and 3 are based on the oxa-23, 24-OXA and OXA-51 sequences" (Turton et al., 2006). OXA-51 like enzymes are present in all *Acinetobacter baumannii* strains tested, and possibly an inherent part of the chromosome (Hériter et al., 2005). OXA-58, less that 50% similar to additional members located in subgroup 4 alone and was found in *Acinetobacter* species from France, Argentina, Turkey, Italy and Romania (Hériter et al, 2005; Coelho et al., 2006; Marque et al., 2005; Vahaboglu et al, 2006; Poirel et al., 2005). "OXA-55 and OXA-SHE, both from *Shewanella algae*, form the fifth group" (Heritier et al., 2004).

The sixth subgroup composed of oxa-48, together with OXA-54 and other oxacillinases originate in the bacteria of environmental origin in Shewanella species

(Poirel et al. 2004 a). “Compared to the rapid increase in global reports on the OXA expression of *Acinetobacter* strains, OXA-48 was detected in a clinical isolate of *Klebsiella pneumoniae* from Turkey” (Poirel et al., 2004b). This variant was resided on the plasmid and has less than 50% amino acid identity to the additional OXA-members.

“The seventh group constituted of OXA-50 enzymes in *Pseudomonas aeruginosa* and contain a set of enzymes that are known as poxB enzymes. It has been reported that many poxB oxacillinase are present on chromosomes in strains of *Pseudomonas aeruginosa*” (Kong et al., 2005). These are designed to be a component of the inherent component of beta-lactamases in that species, but they may possibly not revealed in all the strains and do not results in carbapenem resistance (Girlisch et al., 2004 a). Sub-group 8 comprises OXA-60 a species specific, considered an innate part of the Ralstonia pickettii genome (Girlisch et al., 2004 b) and the subgroup 9 i.e.oxa-62, also known as species specific oxacillinase in *Pandoraea pnomenusa* (Scheider et al., 2006).

The most recent and concerning development is the continuous rise in OXA-48 in enterobacteriaceae particularly in *Klebsiella pneumoniae*. OXA-48 is a weak carbapenemase and like many weak carbapenemases, relies upon other combinational mechanisms like outer membrane mutations and efflux pumps to mediate resistance against carbapenems (Gulmez et al., 2008). Although OXA-48 does not appear to be as productive as KPC-type enzymes, there are not only the cases of the Mediterranean, including Tunisia and Turkey (Cuzon et al., 2010; Carrer et al., 2010). India too has OXA-48 type enzymes among *Klebsiella* species (Sharma et al., 2016).

“OXA-48, a plasmid residing gene, gene shares not more than 50% amino acid identity with the rest of the OXA members”(Dimou et al., 2012). It was transferred to many other members of the species of Enterobacteriaceae through horizontal gene transfer (Dimou et al., 2012). It was revealed in a clinical *Klebsiella pneumoniae* recovered from

Istanbul, Turkey through the capacity of penicillins, imipenem and expanded-spectrum cephalosporins (Poirel et al., 2004 b).

There are eight different OXA-48 variants with some carbapenemase activity; OXA-162, OXA-163, OXA-181, OXA-204, OXA-232, OXA-244, OXA-245 and OXA-370. The first one is OXA-162 exhibits single amino acid substitution when compared to OXA-48. It was found in Turkey from *Klebsiella pneumoniae* isolate. Then, OXA-163 was found in Argentina and it is different from OXA-48 by four amino acid deletions and a substitution (Poirel et al., 2012 b).

The OXA-181 exhibits four amino acid substitutions when compared to OXA-48. It was identified in India, New Zealand, The Netherlands and the Sultanate of Oman from different enterobacteria (Poirel et al., 2012 b). In some cases it was linked to other carbapenemase genes such as blaNDM-1 and blaVIM-5 (Castanheira et al., 2011; Dortet et al., 2012).

“OXA-232, a point mutant derivative of OXA-181, different from OXA-48 by five amino acid substitutions. It is found in France from patients who have been visited to Mauritius or India” (Poirel et al., 2012 b). Another OXA-48 variant is OXA-204 that differs from OXA-48 by two amino acid substitutions and it was identified in patients having connection to Algeria or Tunisia (Poirel et al., 2012 b).

Other OXA enzymes like OXA-244 and OXA-245 were identified in Spain. They have single amino acid substitutions like Arg-222→Gly and Glu-125→Tyr respectively when compared to OXA-48 (Oteo et al., 2013). Furthermore, OXA-370 is different by amino acid substitution that resulted from three nucleotides differences from OXA-48 (Sampaio et al., 2014).

2.7. Non-enzymatic (Efflux pump) resistance mechanism

"Antibiotic efflux as a mechanism for bacterial resistance was first discovered and studied physiologically by Levy" (Levy, 1992). This discovery was demonstrated against tetracycline in enterobacteria. "In his study, Levy observed that tetracycline was expelled from the bacterial cell thus diluting the intracellular concentration of inhibitory agent from the cytoplasm where the target of the drug, ribosome resides and therefore confer tetracycline resistance upon bacterium. Subsequently, it was later found that the tetracycline efflux pump was driven by proton motive force" (Levy, 1992).

After that, efflux mediated resistance is more and more documented as significant means of resistance of bacterial resistance to antimicrobial. Efflux pumps as element of either an acquired or inherent resistance phenotype are proficient of expelling out a large range of substrates from the periplasm to the nearby setting (Poole, 2004).

Principally, efflux pumps are energy reliant conveyor, which pumps out harmful compounds, with antimicrobial agents. "Efflux systems consist of three proteins parts, an energy-dependent pump in the cytoplasmic membrane, an outer membrane porin and a linker binding protein, which binds the two membrane components together" (Nikaido et al., 1998).

Efflux pumps showed distinct substrate specificities; i.e. particular for an antibiotic or a set of antibiotics, while multi-drug efflux pumps have the ability to pump out more than a set of antibiotics (Poole, 2007). The multi-drug efflux systems are of highest significance as they can make the bacterium cell resistant by means of a phenotype of multiple drug resistance besides to the support of cross resistance among the antibiotics and other antimicrobial compounds which are normally used to avoid and control of hospital infections (Piddock, 2006).

"Efflux pumps can be classified into two main classes: ATP-binding cassette (ABC) transporters and secondary multidrug transporters. The source of the energy required for the transport is the major difference between the two classes. Thus, the ABC-type systems use the energy derived from ATP hydrolysis, whereas secondary transporters utilize proton motive force. These ABC pumps involved in drug resistance are mainly drug-specific transporters. Secondary multidrug transporters, which included the majority of clinically relevant efflux systems, based primarily on homology at the levels of primary and secondary structures can be subdivided into four superfamilies. These superfamilies are the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, and the resistance-nodulation-cell division (RND) superfamily" (Fernandez & Hancock, 2012).

The ABC families comprise transporters concerned in both influx and efflux. These carriers can present a broad array of substrates, with sugars, amino acids, ions, drugs, polysaccharides and proteins. These systems consist of transmembrane and nucleotides binding domains that may be present in the same or in separate binding proteins (Moussatova et al., 2008). "Permeases that form a pore through the cytoplasmic membrane usually have six transmembrane regions and they have a tendency to be connected in dimers. There are very few transport of this type, which are concerned in resistance to antibiotics, with the lately recognized MacAB pump, which is involved in the specific macrolide resistance in *Escherichia coli*" (Kobayashi et al., 2001).

SMR family members are small proteins of about 107 to 110 residues. Each of these proteins have four transmembrane segments and normally form tetramers in the cytoplasmic membrane. The number of SMR carrier associated with antibiotics resistance is very small. Examples of proteins from this group are EmrE from *Escherichia coli* (Srinivasan et al., 2009) and AbeS of *Acientobacter baumannii* (Venkatraman et al., 2002).

"Amongst the secondary transporters the MFS superfamily is the most varied family composed of a large number of proteins. Its members can perform three different functions such as uniport (transport of substrate without coupled ion movement), symport (coupled to the movements of ions in the same direction) or antiport (coupled to the ion movement in the opposite direction)" (Marger & Saier, 1993). MFS proteins may possibly have any 12 or 14 transmembrane regions (Paulsen & Skurray, 1993). All MFS transporter concerned in efflux of antibiotics are drugs / proton (H) antiporters (DHAs), and they can be divided into three subfamilies: DHA1, DHA2 and DHA3. The first two subfamilies can pump out several types of antibiotics, and they are present in both prokaryotes and eukaryotes. On the contrary, DHA3 is particularly designed for the pumping out of the antibiotics, such as tetracycline and macrolide, and can only be found both Gram-positive and Gram-negative.

MATE family proteins share a related topology with MFS proteins. "Conversely, they represent a distinct faction because of the low level of homology at the amino sequence level. These proteins comprise 12 transmembrane regions and use sodium gradient for pumping out harmful compounds for instance fluoroquinolones, aminoglycoside and cationic dyes" (Morita et al., 1998). "The members of this family included NorM of *Vibrio parahaemolyticus* and YdhE is homologous to *Escherichia coli* among others" (Morita et al., 1998).

From the clinical point of view, RND is the most relevant efflux pump families that have been best described in Gram-negative bacteria. "These RND efflux pumps consist of three elements: an inner membrane pump protein with 12 transmembrane regions and two large periplasmic loops, a so-called membrane fusion protein, and an outer membrane protein that forms a so called channel-tunnel. The substrates of the RND pumps are very diverse included toxic fatty acids, homoserine lactones, antibiotics, biocides, bile salts,

aromatic hydrocarbons, dyes, inhibitors of fatty acid biosynthesis and detergents. Almost all RND systems are able to pump out multiple antibiotics and coupled this drug efflux with proton antiport. Among the two best-characterized RND pumps are AcrAB-TolC of *E. coli* (Fralick, 1996) and MexAB-OprM of *P. aeruginosa*" (Poole et al., 1993). "However, there are other examples of RND efflux pumps with a demonstrated role in antibiotic resistance" (Fernanadez & Hancock, 2012).

2.7.1. Efflux pumps in *Pseudomonas aeruginosa*

Efflux pump is an important non-enzymatic mechanism of β-lactam resistance in *P. aeruginosa.* Presence of efflux pump also contributes to the development of multiple resistances to all antipseudomonal antibiotics and is mediated by four genetically distinct 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 (Shanti, 2014)

"Cytoplasmic membrane pump	**Periplasmic linker**	**Outer membrane chnel**	**Substrate**
MexB	MexA	OprM	β-lactams including meropenem except imipenem, Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin,
MexD	MexC	OprJ	Meropenem, Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, penicillins except carbenicillin and sulbenicillin, cefepime, cefpirome
MexF	MexE	OprN	Carbapenems, Fluoroquinolones
MexY	MexX	OprM	Meropenem, Quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, aminoglycosides, penicillins except carbenicillin and sulbenicillin, cefepime, cefpirome" (Shanti, 2014)

Table 2: Active efflux systems with their substrates in *P. aeruginosa*

2.7.2. Efflux pump in *Acinetobacter* species:

"The resistance-nodulation-division (RND) family-type pump AdeABC is the best studied efflux pump in *Acinetobacter* thus far and has a substrate profile that includes β-lactams (including carbapenems), aminoglycosides, erythromycin, chloramphenicol, tetracycline, fluoroquinolones, trimethoprim, and ethidium bromide (Peleg et al., 2008). 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" (Shanti, 2014).

2.8. Non enzymatic (Reduced membrane permeability) mechanism

"In Gram-negative bacteria, the outer membrane comprised of an inner layer with phospholipids and an outer layer with lipid A. Such structure reduces drug uptake to a cell and transfer through the outer membrane" (Silhavy et al., 2010). "To gain access, PBP in the inner plasma membrane, β-lactams must either diffuse through or directly traverse porin channel in the outer membrane of Gram-negative bacteria cell wall" (Dwarz & Bonomo, 2010).

"Decreased expression of these outer membrane proteins (OMPs) is another mechanism of resistance. Outer membrane proteins (OMPs) are placed into four large families: general/nonspecific porins, substrate-specific porins, gated porins, and efflux porins" (Martinez, 2008). Porins permit the flow of molecules of ≥1,500 dalton (Hancock

& Brinkman, 2002), whereas general / non-substrate specific, and efflux porins that transmit resistance to carbapenem.

"Some Enterobacteriaceae (e.g., *Enterobacter* spp., *Klebsiella pneumoniae*, and *Escherichia coli*) exhibit resistance to carbapenems based on loss of OMPs; Multiple-drug resistant *Klebsiella pneumoniae* strains exhibit resistant/reduced susceptibility to β-lactams like cephalosporins and carbapenems by the loss of outer membrane proteins known as OmpK35 and OmpK36 together with the production of resistance enzymes, including AmpC β-lactamase and KPC. The loss of Opr D is associated with imipenem resistance and reduced susceptibility to meropenem in the nonfermenter *P. aeruginosa"* (Drawz & Bonomo, 2010). "Resistance to imipenem and meropenem has also been associated with the loss of the CarO OMP in clinical isolates of multidrug-resistant *Acinetobacter baumannii*" (Drawz & Bonomo, 2010).

All carbapenems have different way of interacting with OMPs; a few OMP Influenced by sure carbapenems additional than others plus too the disorder of porin proteins and not at all times enough to set up the resistant phenotype, and that is the means found in association with the expression of β-lactamase (Doumith et al., 2009).

2.9. Carbapenem Resistance-Detection

The efficient, exact and appropriate revealing of carbapenemase-producing organisms is main point of concern not merely for the assortment of suitable guiding principle, however as well for the execution of successful infection procedures. This detection, though, included a series of problems, similar to it cannot just based on the sensitivity report and its valuable procedures has not yet been sufficiently standardized (Miriagou et al., 2010).

In day to day antibiotic susceptibility testing, a decreased susceptibility to the target drugs serve as an major protocol for initial selection of carbapenemase producers which is then followed by tests at the phenotypic and biochemical level. (CohenStuart & Leverstein-Van Hall, 2010). However, carbapenemase gene detection by methods of the molecular level remains the gold standard of detection (Nordmann & Poirel, 2013). Other newer techniques based on newer technologies or analytical methods are also being constantly developed and showed great potential for use due to high efficiency.

Elevation in minimum inhibitory carbapenem (MIC) concentration of carbapenem or reduction in the diameter of the zone of inhibition is the initial reason of suspected carbapenemase in a clinical isolate. This is then followed by more sensitive and specific methods for further testing (Miriagou et al., 2010).

The MIC's of ertapenem are elevated as compared the other carbapenems. Therefore ertapenem seem to be appropriate for detecting carbapenemase production in Enterobacteriaceae family (Nordmann et al., 2012 c). On the other hand, the recognition of carbapenemase producers, base solely on MIC values, can have no sensitivity. Many carbapenemase producing Enterobacteriaceae show broad range of MICs with some values fall within the susceptibility category. Indeed, even sensitivity to carbapenem was seen especially for the OXA-48 / 181 producing enterobacteriaceae which do not have an ESBL (Nordmann, 2010; Nordmann et al., 2011 b).

Although ertapenem does not works well for the cure of infections cause by Acinetobacter and Pseudomonas: (i) it do not have enough antipseudomonal action to be clinically valuable through a grouping of reduced membrane permeability and maybe a increased affinity for efflux pumps (Zhanel et al., 2007); (ii) *Acinetobacter baumannii* strains have a less significant number and range of porins contrast to accompanying Gram-

negative organisms that give to natural outer membrane permeability to ertapenem (Abbott et al. Al., 2013).

"Despite the fact that disc diffusion and MIC determination is suitable and appropriate for the clinical laboratory, but special care should be taken while preparing bacterial inocula" (CLSI). Hence inoculation should be performed adequately and accurately for susceptibility testing to be reliable; if the proper inoculum cannot be assured using an inoculation loop method, inocula should be prepared using standardized Clinical laboratory standard institute (CLSI) guidelines.

2.9.1. Phenotypic methods for carbapenemase detection

Phenotypic, culture-based methods are options that avoid interruption in reports of such strains to the clinic provided molecular tests are not readily accessible. These phenotypic tests included the modified Hodge test, inhibitor-based tests, and the use of specific culture media.

2.9.1.1. Modified Hodge test

"The clover leaf technique, or modified Hodge test, is available in routine clinical settings and has been extensively used as a most common phenotypic method to detect carbapenemase activity" (CLSI).

"The modified Hodge test is performed on Mueller–Hinton agar" (CLSI). "The principle is based upon inactivation of a carbapenem by a carbapenemase-producing test strain" (CLSI). "An indicator organism, usually *Escherichia coli* ATCC 25922 at a turbidity of 0.5 McFarland standards, is used to inoculate the plate surface, and a carbapenem disc is placed at the centre. "After overnight incubation, the cloverleaf-shaped indentation of growth of the test strain versus the susceptible indicator strain is interpreted as a positive result for carbapenemase production by the tested strain" (CLSI).

The major advantage of the modified Hodge test is that it provides excellent sensitivity for Ambler class A and class D carbapenemases detection. In this manner it is helpful in recognitions of enzymes with weak carbapenemase activity for instance OXA-23, GES-5 and GES-6 (Vourli et al., 2004)**.** While looking for different methods, the modified Hodge test used in combination to carbapenem susceptibility testing was used to confirm KPC-producers among Enterobacteriaceae isolates (Anderson et al., 2007).

Modified Hodge test lacks sensitivity for the detection of MBL-producers (Thomson, 2010). In order to improve the detection limits for MBL especially NDM-producers, the authors suggested for the addition of zinc sulphate (100 µg/mL) to the Muller–Hinton agar media. Since MBL activity is dependent on zinc the above modification increased sensitivity of the test to 85.7%. "In the same way, when detecting MBLs of *Pseudomonas aeruginosa* and *Acinetobacter* by the modified Hodge test, Lee et al. also showed that the test can be improved by using an imipenem disc to which 10 µL of 50 mM zinc sulfate (140 µg/disc) has been added or by using Mueller–Hinton agar to which zinc sulfate has been added to a final concentration of 70 µg/ml" (Lee et al., 2003).

The "Modified Hodge test" also has more shortcomings. These shortcomings involve the carbapenem source best for carbapenemase detection, where imipenem look to be the least particular way yet even it is mostly sensitive to detect OXA-48 enzymes. "In addition, the modified Hodge test may not have a sensitivity to detect carbapenemase action in *Enterobacter* species" (Nordmann and Poirel, 2013). In addition, false positive results are anticipated with the AmpC producers, rather than with imipenem than another carbapenem (Thomson, 2010).

In summary, the modified Hodge test is a simple, cost effective phenotypic method that is able to apply through the screening of carbapenemases subsequent to antimicrobial susceptibility testing. It possibly helpful to make use of this test in the infection control

practice as an extra approach that will help in controlling carbapenemase activity in outbreaks.

2.9.1.2. Inhibitor based method

"Phenotypic tests involving these inhibitors are based upon in vitro observation of an increase in inhibition zone diameter (or reduction of the MIC) of the tested isolate in the presence of a carbapenem combined with a carbapenemase inhibitor compared to the same carbapenem alone" (Hammoudi et al., 2014).

The occurrence of synergy involving carbapenem and the inhibitor can be basically demonstrated by various methods.

In the combined disc test, the inhibitor is supplemented to a commercially presented disc of carbapenem; "the combined disc and a disc of the same carbapenem are placed on Muller–Hinton agar streaked with the test strain" (Pasteran et al., 2009). The combination disc can be either prepared in-house and dried in air or purchased as a ready combination tablet or Neo Sensitabs (Rosco Diagnostica, Denmark).

In agar dilution assays, an increase in carbapenem inhibition zone diameter or observation of the reduction in carbapenem MIC, or, in the presence of an inhibitor in the culture medium is also a positive test for carbapenemases (Pasteran et al., 2009).

Inhibitors of class A carbapenemases

Phenotypic tests are designed to identify Class A- carbapenemase producing strain in the presence of boronic acid compounds, typically 3 aminophenylboronic acid (APBA). These compounds are active site-directed serine β-lactamase inhibitors, and in contrast to clavulanate and tazobactam are not based on a β-lactam structure.

Actually Class A carbapenemases are not exactly identified by inhibitors such as clavulanate, tazobactam, and boronic acids acid compounds signify a more sensitive means

of inhibition (Thomson, 2010). “It is reported that an increase in inhibition zone diameter by 4–7 mm with carbapenem discs i.e. meropenem discs with or without 400 µg of APBA is a cut-off value for the production of class A carbapenemases” (Tsakris et al., 2009).

“In tests utilizing MIC, a three-fold or greater reduction of MIC of the carbapenem in the presence of 0.3 g/L of APBA was proposed as another cut-off value” (Pasteran et al., 2009).

In terms of combined discs strategy, synergy between boronic acid and carbapenems was used to detect the first KPC-producing *K. pneumoniae* in a tertiary care hospital in Greece (Tsakris et al., 2008). Shortly Tsakris et al. noted boronic acid synergy with imipenem or meropenem by means of combined disk tests for a big group of K. pneumoniae producing KPC through distinct array of carbapenem MICs.

In summary, it is reported that testing of boronic acid tests are precise for KPC when they are performed with Imipenem or meropenem, but with ertapenem in an strain co-produce a plasmid residing AmpC-β-lactamase (Tsakris et al., 2009). Other authors reported that boronic acid compounds exhibit potent activity against a wide range of β-lactamase classes and may results in difficulties in the detection of carbapenemases (Pournaras et al., 2010). “AmpC producers are sensitive to APBA, but are differentiated by inactivation Cloxacillin, a commonly used AmpC inhibitor” (Giske et al., 2011).

In the strain possessing VIM and KPC carbapenemase, it is to state that the assays based on APBA, failed to detect KPC production (Giakkoupi et al. Al., 2009)

Hence, inhibition assays with APBA panels combined with imipenem & meropenem possibly handy for class A carbapenemase detection although its specificity is verified by susceptibility tests (Miriagou et al. 2010).

Inhibitors of class B carbapenemases (Metallo-β-lactamses)

All metallo-beta-lactamases (MBL) often involve zinc ions as metal cofactors for their enzyme activity and, consequently, affected by the removal of zinc from its active ingredient (Walsh et al., 2005). Studies reporting MBL detection have depend upon this principle, and a number of inhibitors that take advantage of metallo enzyme inhibition has been considered. These inhibitors have ethylenediaminetetraacetic acid (EDTA), EDTA plus 1,10-phenanthroline, thiol compounds such as 2-mercaptopropionic acid or sodium acid thioglycolic and dipicolinic acid.

"Detection depends on the synergy between these inhibitors and a carbapenem (Imipenem and/or meropenem) and/or an oximiminocephalosporin (ceftazidime)" (Miriagou et al. Al., 2010). Conversely, the differences in inhibition of these compounds and MBL are not continuous in their resilience to the generally used screening compounds, such as imipenem and ceftazidime. Hence, there is no specific inhibitor that can identify all MBL, and some of these carbapenemases can be fail to spotted, particularly in carbapenem intermediate/sensitive Enterobacteriaceae (Walsh et al., 2005).

Lee et al. applied the double disc synergy test to find out synergy between imipenem and a set of MBL inhibitors including EDTA and thiol compounds like mercaptopropionic acid and sodium mercaptoacetic acid, in IMP-1 and VIM-2 producing *Pseudomonas* and *Acinetobacter* strains. EDTA disks were more precise in detection of MBL-producing P. aeruginosa strains, whereas in Acinetobacter spp. mercapto propionic acid and sodium mercapto acetic acid have been functional which explained that the thiol compounds may be more useful to decrease the false detection of positive MBL in *Acinetobacter* spp. EDTA and sodium mercaptoacetic acid combined discs showed higher sensitivity in compared to single inhibitor discs with both organisms (Lee et al., 2003).

“In one study with 27 NDM-positive enterobacterial isolates, 25 isolates were positive in the imipenem/imipenem-EDTA Etest strip, while all 27 isolates were positive in a combined disc of EDTA and imipenem” (Nordmann et al., 2011 a). “The authors reported that the combined disc method is to be applied for routine detection of MBL producers, while the combined Etest is more suitable for laboratories which do not screen MBL on a daily basis, or which perform susceptibility testing in liquid media and rarely apply disc diffusion techniques” (Nordmann et al., 2011 a).

In *Acinetobacter baumannii*, some studies have reported failure of EDTA inhibition tests to detect MBLs (Ikonomidis et al., 2008; Loli et al., 2008), while others have showed chances of false positive results in isolates that produce OXA-23, due to higher possibility of conversion of this enzyme into a less active monomeric form in presence of EDTA (Segal & Elisha, 2005). “Besides, it has been noted that EDTA may inhibit some bacteria solitary, which leads to false positive results due to an increase in the permeability of the outer membrane” (Chu et al., 2005). In general, it is necessary to test the intrinsic activity of the chelators alone to avoid false positive results in case it inhibits the tested isolate (Ratkai et al., 2009).

Sensitivity of MBL production by various phenotypic tests like Etest, double disc and combined disc test with imipenem and EDTA has enhanced through the incorporation of zinc to the culture media. This has been improved due to possible formation of functional MBL molecules in the periplasmic space of bacteria, since these enzymes depend on zinc for their activity. In addition, the comparatively elevated Zn absorptions in growth decrease the expression of *Pseudomonas aeruginosa* porins such as OprD, and consequently, rates of diffusion of carbapenem, thus further improving activity of carbapenemase (Sekyere et al., 2015).

On the other hand, even out of these inhibitor-based detection tests are not yet done, since there is no single inhibitor that shows effective to all types of carbapenem-resistant bacteria. The selection of the accurate inhibitor and methodology for the detection of MBL, therefore, should be performed depending upon the nature of MBL producers in a type of the hospital setting (Hammoudi et al., 2014).

Inhibitors of Class C carbapenemases

This class of enzymes have an effect on a wide range of β-lactams such as penicillins, oxyiminocephalosporins, cephamycins and aztreonam, excluding ACC that does not confer resistance to cephamycins and is inhibited by cefoxitin (Girlich et al., 2000). Because they may include the AmpC cephalosporinases which is resided on the plasmid such as DHA, the ACC and CMY to be present in non susceptible carbapenem strain with another means of resistance, therefore, add to a bigger danger of poor health results (Thomson, 2010). This is particularly significant in isolates such as *Klebsiella*, *Salmonella* and *Proteus mirabilis*, which do not produce chromosomal AmpC cephalosporinases.

The two most commonly used inhibitor of AmpC are boronic acid & cloxacillin. On inhibition by the above mentioned two inhibitors, they increase the cephalosporin activity (Jacoby, 2009). Boronic acid inhibition tests were also useful to detect inducible and non inducible AmpC cephalosporinases in *Pseudomonas aeruginosa* (Upadhyay et al., 2011). However, these inhibition tests need careful interpretation. Because boronic acid inhibits KPC enzymes, some ESBLs and OXA-12, and may inhibit the growth of certain bacterial strains (Mammeri et al., 2008; Thomson, 2010). Inhibition of AmpC cephalosporinases by means of oxacillin can be done also by adding cloxacillin powder in the culture medium otherwise by the use of cloxacillin disc.

Inhibitors of Class D carbapenemases

"Class D carbapenemases are generally not inhibited by clavulanic acid, tazobactam, sulbactam, cloxacillin, boronic acid compounds or zinc chelators" (Poirel et al., 2010). It has been seen that the activities of class D β-lactamases may be inhibited in vitro by sodium chloride (NaCl) at a 100mM concentration (Poirel et al., 2010). Because of the fact that this characteristic is not shared among other β-lactamases, it acts as a key attribute for in vitro detection. On the other hand, this feature has not been used in a normal laboratory setting for carbapenemase detection.

2.9.1.3. KPC+MBL detection commercial kit

"Rosco Diagnostica has launched a kit in the form of tablets that are used for qualitative in vitro identification of microbial resistance mechanisms by the agar tablet/disc diffusion method, in order to confirm the mechanism by which the organism has gained resistance to specific antimicrobial agents in Enterobacteriaceae" (Rosco Diagnostica).

Principle:

Four cartridges of Neo-Sensitabs having meropenem with three other cartridges have inhibitors of different β-lactamases. Following were probable explanation for the strain expressing reduced carbapenem susceptibility:

1-"The organism hyper-produces AmpC. Because of the slow hydrolyses of carbapenems by the AmpC enzyme, the AmpC is probably coupled to other resistance mechanism like efflux pumps, porin loss or other β-lactamases. The AmpC enzyme is inhibited by cloxacillin. The cloxacillin is used to distinguish between AmpC and KPC since both are inhibited by Boronic Acid" (Rosco Diagnostica).

2- "The organism produces a Metallo β-lactamase that hydrolyses carbapenems efficiently. MBLs are inhibited by dipicolinic acid. DPA has no (as opposed to EDTA) intrinsic

antimicrobial activity and thus the results with this compound are more easily interpret" (Rosco Diagnostica).

3- "The organism produces a KPC enzyme. KPC enzymes are inhibited by boronic acid. However, Boronic Acid also inhibits the AmpC and in order to raise the specificity of the Kit, the cloxacillin combination is included to distinguish between the two" (Rosco Diagnostica).

2.9.1.4. Culture media for detection of carbapenemase

There are several commercially available media for the detection of carbapenemase producing isolates. Examples included, CHROMagar Acinetobacter (CHROMagar, Paris, France), Brilliance CRE medium (Oxoid, Thermofisher Scientific, Illkick, France), CHROMagar KPC (CHROMagar, Paris, France), chromID Carba (bioMérieux, La Balme-les-Grottes, France) and SUPERCARBA medium.

"Commercially available carbapenem containing media has drawback that these are not able to differentiate between carbapenem resistance due to inactivation by enzymes or due to other resistance mechanisms" (Moran Gilad et al., 2011). Although the SUPERCARBA medium has small benefit over other means given that supposedly it hampers carbapenem resistant but not carbapenemase producing organisms (Nordmann et al., 2012 b).

2.9.1.5. Carbapenemase detection by rapid colorimetric methods

Carba Nordmann-Poirel (NP) test

"This method is used for the detection of carbapenemases in Enterobacteriaceae and Pseudomonas. This method is based upon hydrolysis of the β-lactam ring of imipenem by the tested strain, followed by colour change of a pH indicator, usually phenol red from red to yellow/orange" (Nordmann et al., 2012 d). "This test differentiates carbapenemase

producers from isolates resistant through other mechanisms, such as outer membrane permeability and/or production of cephalosporinases and/or ESBLs as well as from carbapenem susceptible strains with non-carbapenem hydrolyzing ESBL, plasmid encoded or chromosome-encoded cephalosporinases" (Nordmann et al., 2012 d).

The test requires less than 2 hours, and eliminates the need of other in vitro phenotypic tests like the modified Hodge test or the inhibitor-based techniques. This test is very useful in contrast to the genetic methods in detecting not just all recognized carbapenemases (Ambler classes A, B, and D), however also identifies novel and emerging carbapenemase also. It is also cheap and has no requirement for special equipment (Nordmann and Poirel, 2013). On the other hand, the above mentioned does not detect carbapenemases with weak activity such as GES-type.

"A latest study illustrate a new form of the Carba NP test, the CarbAcineto NP test, proposed for carbapenemases detection in *Acinetobacter* spp. with the same principle as in the original test, the CarbAcineto NP test utilizes modified lysis conditions and an increased inoculum size" (Dortet et al., 2014). "In contrast to the Carba NP test that identify only metallo-β-lactamases in *Acinetobacter* spp., this newly proposed test can detect all carbapenemase types in this organism and its sensitivity and specificity are very high" (Dortet et al., 2014).

Blue Carba test

More recently, Pires et al., in 2013 proposed the Blue Carba test based on the same principle. In this test variant, bromothymol blue was used as indicator instead of phenol red, since the pH range (6.0 to 7.6) of the former overlaps the optimum pH of pH 6.8 of most β-lactamases (pH 6.8). The increased sensitivity obtained thereby allowed for a direct colony approach without the intermediate step of cell lysis. This Blue carba test is helpful

for the of carbapenemase detection in Enterobacteriaceae, *Acinetobacter* & *Pseudomonas aeruginosa.*

Rosco CARB Blue kit

This kit includes imipenem(x2)+bromothymol blue for the carbapenemase detection on the basis of hydrolysis of beta lactam ring which results in the change in the pH and cause the change of colour of indicator. Its detection of OXA-48 producers and inadequately hydrolytic carbapenemases are useless and not supposed to be used in regions with high OXA-48 producer (Miriagou et al., 2013, Yusuf et al., 2014).

Neo-Rapid CARB Kit

"This commercial Neo-rapid CARB kit based on the similar principle of β-lactam ring hydrolysis of a carbapenem is applied to the Enterobacteriaceae and *Pseudomonas aeruginosa* rapid detection of carbapenemase" (Rosco Diagnostica). This test is performed quickly and the reading of the results is ready within 30 minutes to 1 hour from the time the reaction is started. Thus applying this kit in the routine screening of carbapenemase saves time and efforts in the laboratory.

2.9.1.6. Carbapenem Inactivation Method (CIM)

"A new phenotypic test, known as Carbapenem Inactivation Method (CIM), was recently developed to detect carbapenemase activity in Gram-negative rods within eight hours". (Zwaluw et al., 2015). "The CIM is the first to use antibiotic susceptibility-testing disks, which are globally available at low cost and have long storage lives, as substrate aliquots for this. This greatly improves practicality and reduces costs and labour. In addition, this method was shown to be unaffected by changes in variables such as incubation temperature or time, disk manufacturer, laboratory staff and the age of the culture or bacterial suspension making it a cost-effective and highly effective phenotypic

screening method that can reliably detect carbapenemase activity. Furthermore as it requires no specialized equipment, reagents or skill and low hands-on time to perform, it is a high throughput method allowing screening of large numbers of bacterial isolates in most normal setting microbiological laboratories" (Zwaluw et al., 2015).

2.9.2. Additional tests

The Spectrophotometric analysis of hydrolysis of carbapenem is measured to be quality method for detection of carbapenemase in a doubtful carbapenemase producing strain. Utilizing the crude cell extracts in the presence/absence of inhibitors such as EDTA for MBL, clavulanic/tazobactam for KPC & NaCl for OXA type for hydrolysis of carbapenem gives extra information on the type of enzyme. But at the same time, these are technically demanding also (Shanti, 2014).

"Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) has been recently introduced into the diagnostic microbiology laboratory" (Nordmann and Poirel, 2013). "The basic MALDI-TOF MS principle relies upon the detection of antibiotic degradation products by bacterial hydrolyzing enzymes, mixed with a suitable matrix which has different mass spectra than both the antibiotic and the degradation product" (Hrabak et al., 2013). Another advantage of the MALDI-TOF MS is reduction of the turnover time needed to obtain results till an average of about 4 hour if a fresh bacterial culture is available the interpretation of the spectra needs special skills, and the identification of the carbapenemase type is not possible.

IEF i.e. isoelectric focusing distinguishes proteins by charge, and the recognition of β-Lactamases is attained by means of the chromogenic cephalosporin nitrocefin. While IEF outcomes may not recognize a specific β-lactamase, isoelectric point information about IEP & inhibition features can be gettable by this process. IEF is particularly useful for

detection of several β-lactamases in one isolate. “IEF can also be combined with a bioassay to detect the presence of carbapenemases using an overlap of agar with imipenem and a second overlap with a susceptible indicator organism. Growth through an enzyme band indicates a potential carbapenemase” (Shanti, 2014).

2.9.3. Analysis of carbapenemase genes by Genotypic means

Polymerase chain reaction is nowadays has become a normal routine method in many clinical laboratories to overcome the problems associated with phenotypic detection (Miriagou et al., 2010). PCR technology carried out on genomic DNA can produce results inside 4-6 hours, and perhaps next by sequencing if necessary, to accurately identify a carbapenemase types, relatively than just their group (e.g. VIM-type, KPC-type, NDM-type, and OXA-type) (Poirel et al., 2011 b).

There are different types of PCR. It can be single, multiplex, or real-time and a set of recently published primers and standardized cycling conditions are now available which allow easy and useful detection of clinically significant carbapenemase genes (Dallenne et al., 2010).

In addition to “in-house” assays, commercially available PCR kits are also available. Check-MDR CT102 DNA microarray is one such kit which are based on DNA amplification then followed by amplicon detection in a tube microarray. This allows detection of multiple genes in a single test.

In one another type of commercial amplification kits, enzyme-linked immunosorbent assay (ELISA) follows carbapenemase gene amplification (Gazin et al., 2012). In the hyplex®-MBL ID Multiplex PCR-ELISA, bacterial DNA is amplified by multiplex PCR, immobilized on polystyrene-ELISA plates, hybridized to specific oligonucleotide probes, and the hybridized complexes are detected by a peroxidase

conjugated antibody resulting in change of color. “In a Greek study, this above mentioned method showed high 98% sensitivity and high 98.6% specificity in detection of VIM producers among *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacteriaceae” (Avlami et al., 2010).

One another method is loop-mediated isothermal amplification (LAMP) method. It can be used for the fast and responsive detection of NDM gene (Qi et al., 2012). In comparison to PCR, LAMP is more specific and takes less time to perform. “In addition to this, gel electrophoresis is not needed, because the above method synthesizes large amounts of DNA where products can easily be detected by turbidity or fluorescence (Chen & Cui, 2009). LAMP method showed a potential and important and valuable means for detection of NDM-1 gene” (Qi et al., 2012).

The use of a RT (real time) PCR assay which applied fluorescence resonance energy transfer (FRET) hybridization probe based detection of KPC and NDM encoding genes simultaneously. Also, another real time PCR with SYBR Green detection was developed for screening of KPC producing organisms in stool specimens and nasal swabs. An internal control is included in this assay in order to avoid inhibition of PCR in these specimens. The KPC specific primers amplified the internal control. This assay was sensitive and specific, can be performed in less than 2 hour time period, and is at least two-times less expensive than real time PCR with probes, due to the low cost of the fluorescent molecule SYBR Green (Wang et al., 2012).

High cost, the requirement of high skill and lack of ability to identify novel unidentified genes are the key limitations of the PCR methods (Nordmann et al. 2012 c). The high diversity of carbapenemase-encoding genes and the increasing number of new variants imply that a negative PCR result concerning a carbapenem resistant strain in a

local laboratory setting requires re-evaluation by a reference laboratory for further genotypic analysis (Cohen Stuart & Leverstein-Van Hall, 2010)

2.9.4. Efflux pump: Detection Methods

There are different strategies which are used to counteract efflux-mediated resistance which are based on the search of either new antibiotics bypassing the efflux systems or efflux pump inhibitors (EPIs). There are different ways to reach these objectives either by improving the molecular design of old antibiotics to reduce their efflux, or by blocking pump function (Amaral et al., 2014). Following may be considered as different means of efflux pump inhibitors:

(1) "competing with antibiotics or other substrates for access to the EP" (example, Phe-Arg-β-naphthylamide PAβN; Martins et al., 2009)

(2) "reduced access to ions such as calcium (Martins et al., 2011) needed by ancillary components of the pump, possibly those which induce conformational changes of the fusion proteins, needed to produce the peristaltic activity that also promotes the movement of water and compounds through the efflux pump" (Seeger et al., 2008),

(3) "Inhibiting access to the energy provided by the proton motive force. For example, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)"; (Varga et al., 2012);

(4) "Inhibition of metabolic enzymes that yield hydronium ions needed for the maintenance of the proton motive force" (Amaral et al., 2011a);

(5) "Simply by non-specific blocking (coating) of the bacterial envelope" (Amaral & Kristiansen, 2000).

On the other hand the easy technique is to display an over expressed efflux pump system of pathogenic bacteria involved 24-hr old culture of MDR isolate and reference

strains representing the wild-type, and their equivalents is the ethidium bromide-agar method.

In the ethidium bromide cartwheel method, the minimum concentration of etbr that shows fluorescence is noted. On the other hand, the wild type equivalent reference strain is beginning to produce signs of fluorescence at a given low concentration of Etbr, The MDR clinical strain, the MDR phenotype of whom is because of mainly or in part, to a over expressed efflux pump begins to show fluorescence at a much higher concentration of Etbr. If performed correctly, because of its simplicity, this method/technique is more or less always successful (Martins et al., 2010).

This method can be valuable for instantaneous demonstration of two strains which differ from each other by the presence or absence of an efflux pump. This method has as well been valuable for indicating plasmids loss that has genes coding for a certain efflux pump (Costa et al., 2010).

An alternative method involving the use of acridine orange as a fluorescent reagent is available for the centers avoiding the use of ethidium bromide (Martins & Amaral, 2012). On plain agar, it may be ethidium bromide/acridine orange as fluorescent agents can be employed to detect the efflux pump system in terms of energy and requirements for various ions.

Irrespective of the usefulness of Etbr cartwheel method, for studying the efflux pump mechanisms, its inability to provide data on real time basis is a major drawback. This was overcome by a advance automated EB method which uses Corbett Research 3000 ™ thermal cycler for assessment of efflux and consideration of agents on a real time basis within defined physiological conditions (Paixao et al., 2009; Viveiros et al., 2010).