PHENOTYPIC AND GENOTYPIC DETECTION OF EXTENDED-SPECTRUM β-LACTAMASES IN CLINICAL ISOLATES OF KLEBSIELLA PNEUMONIAE CARBAPENEMASE-POSSESSING ENTEROBACTERIACEAE

Thesis

Submitted for Partial Fulfillment of Master Degree in Clinical and Chemical Pathology

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2012

INTRODUCTION

Since extended-spectrum β-lactamases (ESBLs) were initially reported to be in *Klebsiella ozaenae* in the first half of the 1980s in Germany, ESBLs have been spreading globally. The Clinical and Laboratory Standards Institute) (CLSI) interpretive guidelines stated that strains of *Klebsiella* spp. and *Escherichia coli* that produce ESBLs may be clinically resistant to therapy with penicillins, cephalosporins, or aztreonam, despite apparent in vitro susceptibility to some of these agents ***(Song et al., 2007).*** For more than two decades, carbapenems have been considered the pharmacotherapy of last resort for managing multidrug-resistant infections caused by *Enterobacteriaceae* ***(Toye et al., 2009).***

During the last decade, carbapenem resistance has emerged among clinical isolates of the *Enterobacteriaceae* family, and this is increasingly attributed to the production of β-lactamases capable of hydrolyzing carbapenems. Among these enzymes, a new type of Ambler class A β-lactamase, the *Klebsiella pneumoniae* carbapenemase (KPC), which has been rapidly spreading among *K. pneumoniae* isolates and other *Enterobacteriaceae* in the northeastern regions of the United States and has spread to several regions of North and South America, as well as in China, and Greece ***(Tsakris et al., 2009a).***

Bacteria producing KPC**s** are rapidly emerging as a cause of multidrug-resistant infections worldwide. Bacterial isolates

harbouring these enzymes are capable of hydrolyzing a broad spectrum of β-lactams including the penicillins, cephalosporins, carbapenems and monobactam ***(Hirsch and Tam, 2010).***

KPC-possessing strains frequently carry ESBL genes, which could possibly contribute to the expression and dissemination of the β-lactam resistance trait. It should be also noted that KPCs and ESBLs are mostly plasmid-encoded determinants that can easily disseminate to other enterobacterial strains. Therefore, the phenotypic detection of ESBLs in KPC-producing isolates of the *Enterobacteriaceae* is of potential interest for epidemiological purposes as well as for limiting the spread of the underlying resistance mechanisms ***(Endimiani et al., 2009)***

The CLSI recommends a phenotypic confirmatory test for ESBL production that consists of measuring the growth inhibitory zones around both cefotaxime (CTX) and ceftazidime (CAZ) disks with or without clavulanic acid (CA) for *K. pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, and *Proteus mirabilis*. Different double-disk synergy tests (DDSTs) based on the synergy of amoxicillin-clavulanate (AMC) with extended-spectrum cephalosporins and aztreonam have also been extensively used for the detection of ESBLs ***(Drieux et al., 2008).***

KPCs hydrolyze several β-lactam antibiotics, and hence, the presence of an ESBL can be masked by the expression of a KPC. Moreover, the weak inhibition of KPCs by the β-lactamase

inhibitors may interfere with the interpretation of ESBL detection methods and KPC enzymes may be mistaken for ESBLs. Thus, there is a need to accurately detect ESBLs in the presence of coexisting KPC expression ***(Petrella et al., 2008).***

Boronic acid (BA) compounds are serine-type β-lactamase inhibitors that were employed originally for the detection of class C plasmidic AmpCs in *Enterobacteriaceae*. Recently, they have also been evaluated for the differentiation of KPC-producing *Enterobacteriaceae* ***(Pournaras et al., 2010).*** BA-based tests with disks of CAZ and CTX have also been successfully employed for the identification of ESBLs in AmpC and KPC producers ***(Tsakris et al., 2009a).***

AIM OF THE WORK

Detection of ESBLs among KPC positive and negative isolates by using boronic acid and PCR for detection of ESBL genes aiming for:

1. Determining frequency of association of ESBLs and KPC in *Enterobacteriaceae*.
2. Evaluating boronic acid in comparison to multiplex PCR (standard method) in detection of ESBL genes (SHV, TEM and CTX-M).

β-LACTAM ANTIBIOTICS

Since the antibiotic properties of penicillin were first noticed in the beginning of last century, β-lactam based antibiotics have been well developed as miracle drugs for the therapy of bacterial infectious diseases in clinics ***(Xing et al., 2008).***

The β-lactam antibiotics in clinical use are penicillins, narrow- and extended-spectrum cephalosporins, monobactams and carbapenems (Figure 1). The common structural feature of these classes of antibiotics is the highly reactive four-membered β-lactam ring ***(Drawz and Bonomo, 2010)*** which may be fused to five- or six-membered heterocyclic rings. Heterocyclic rings may be saturated or unsaturated with a double bond positioned between positions 3 and 4. The heteroatom in position 1 may be sulfur (penams, cephems, and penems), carbon (carbapenems and carbacephems), or oxygen (clavams, oxapenems, and oxacephems). Monobactams, a structurally distinct class of agents, consist of unfused β-lactam rings (Figure 2) ***(Babic, et al., 2006***).

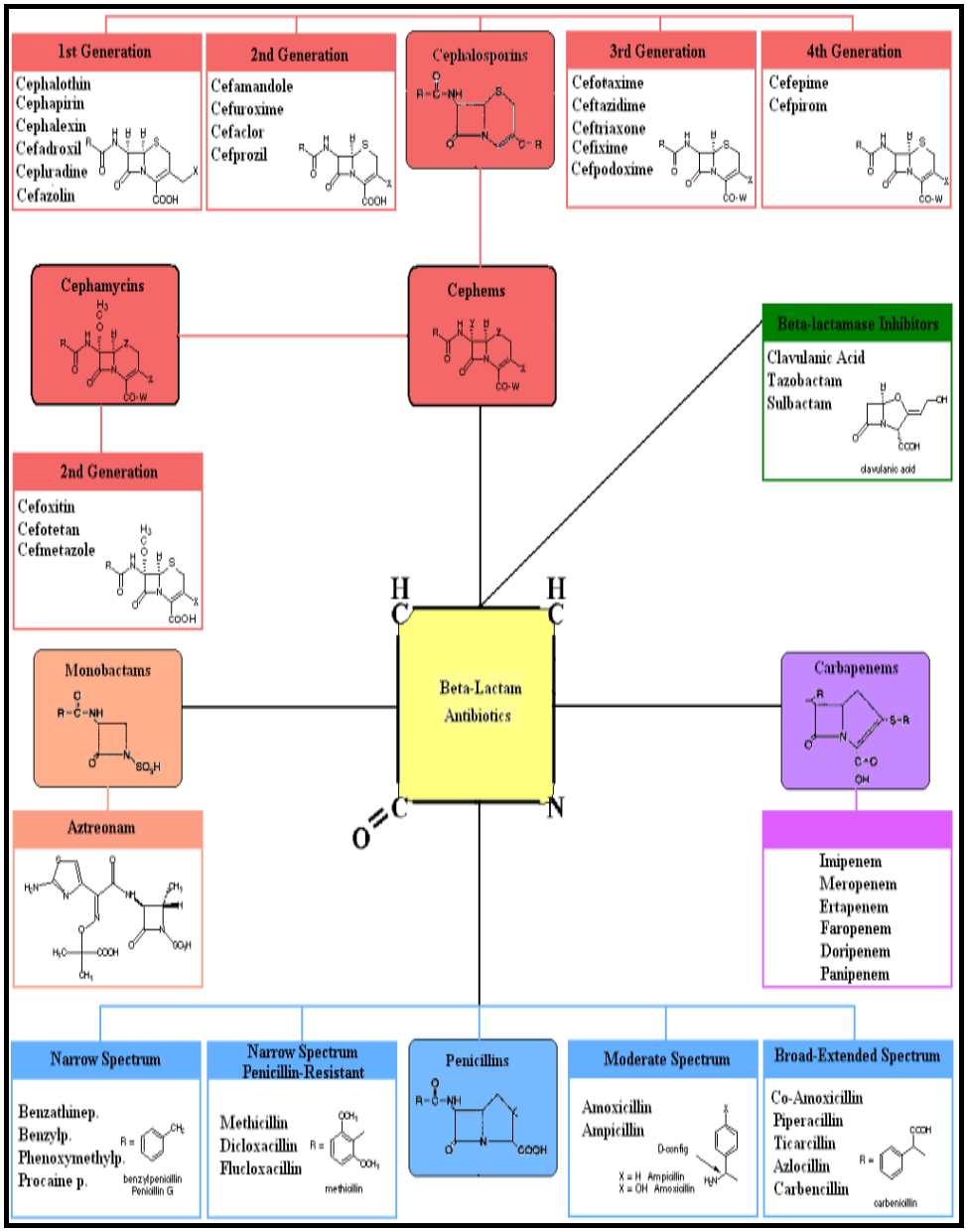

Figure (1): Schematic presentation of β-lactam antibiotics ***(Drawz and Bonomo, 2010).***

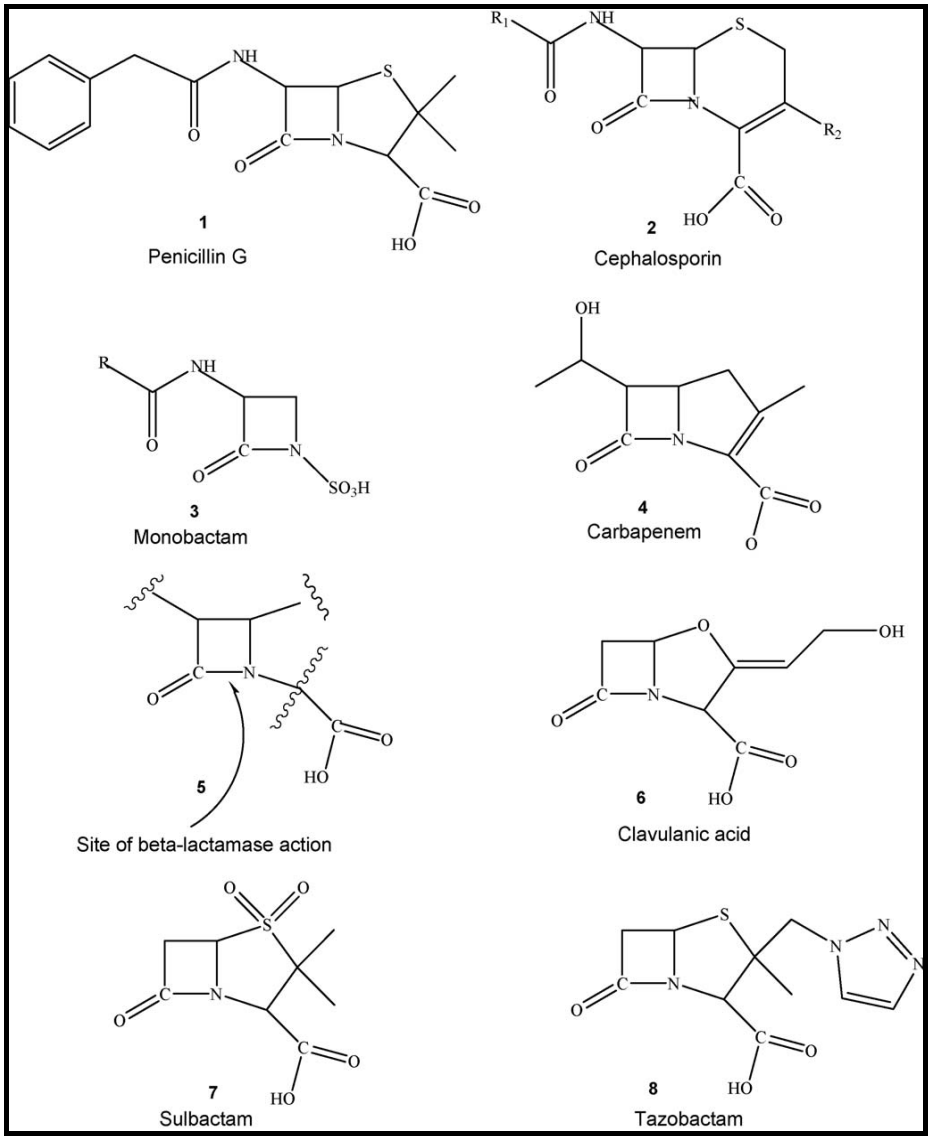

Figure (2): Chemical structures of β-lactams (1-4), site of action of β-lactamases (5), and chemical structures of β-lactamase inhibitors used in clinical practice (6-8) ***(Babic et al., 2006).***

Mechanism of action of β-lactam antibiotics

All β-lactam antibiotics are bactericidal agents that inhibit cell wall synthesis. The bacterial cell wall is a complex structure

composed of a tightly cross-linked peptidoglycan net (Figure 3). The glycan component of this rigid structure consists of alternating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) (Figure 4). This varies among the Gram-negative and Gram-positive species, but always terminates in two D-alanine residues (Figure 5).

Beta-lactams are sterically similar to the penultimate D-AlaD-Ala of the pentapeptide that is attached to NAM; hence PBPs mistakenly use penicillin as a substrate for cell wall synthesis and the transpeptidase (or carboxypeptidase) is acylated. The acylated PBP cannot hydrolyze the β-lactam and subsequent steps in cell wall synthesis are hindered while autolysis by cell wall degrading (autolytic) enzymes continues. Bacterial cells become permeable to water, rapidly take up fluid, and eventually lyse ***(Meroueh et al., 2006)***. However, it was noted that penicillins were able to cause inhibition of growth in certain bacteria without bacteriolysis.

Therefore, triggering of autolytic cell wall enzymes was considered as a second and separate target of β-lactam agents ***(Babic et al., 2006).***

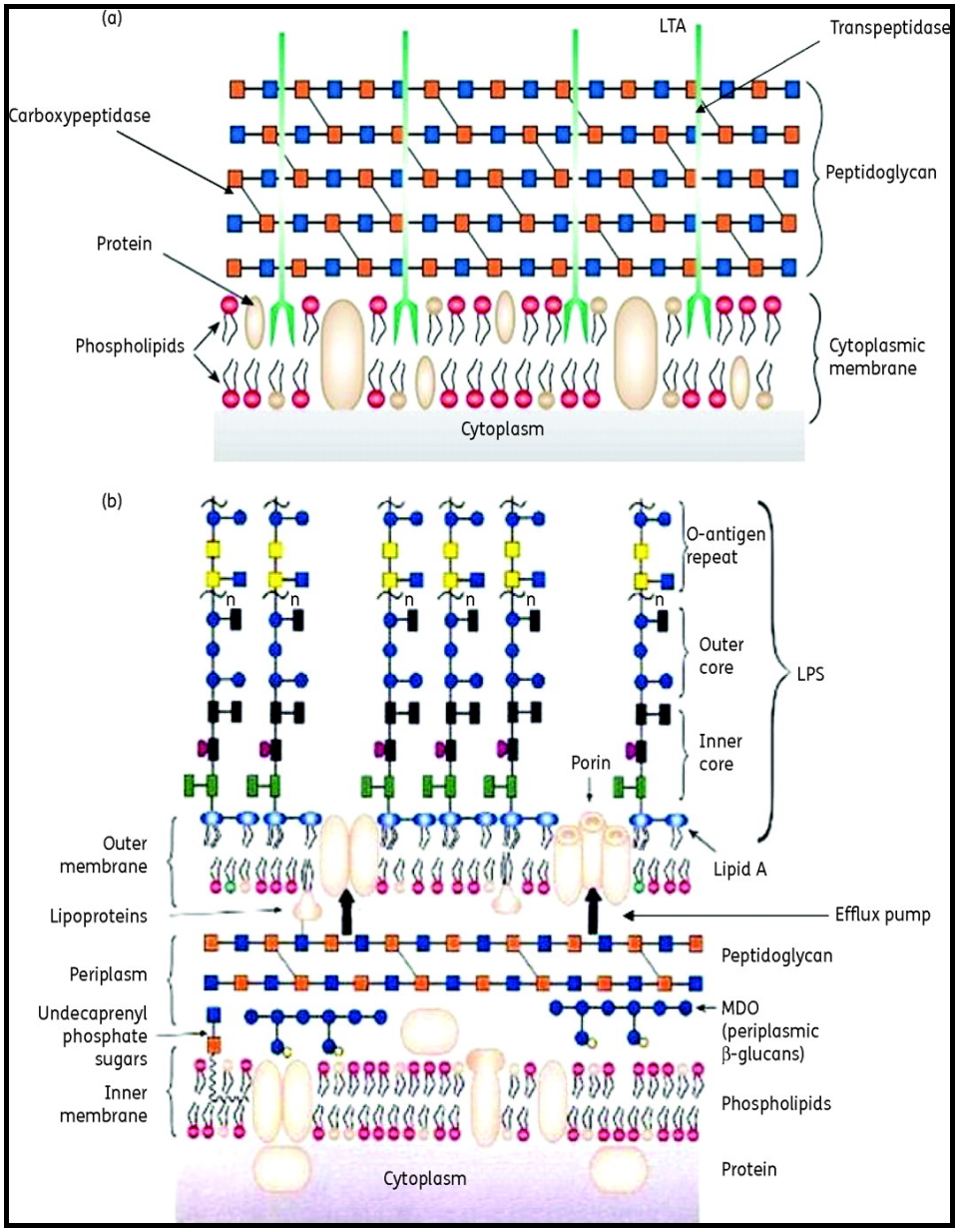

Figure (3): Structure of the cell walls of *S. aureus* (a) and *P. aeruginosa* (b). LTA, lipoteichoic acid; LPS, lipopolysaccharide ***(Meroueh et al., 2006)***.

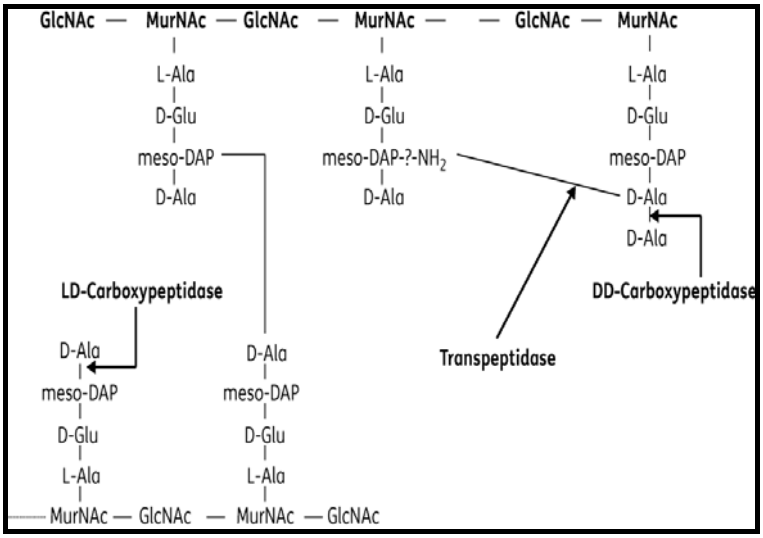

Figure (4): Penicillin-sensitive enzymes involved in cell wall biosynthesis GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; DAP, diaminopimelic acid ***(Meroueh et al., 2006).***

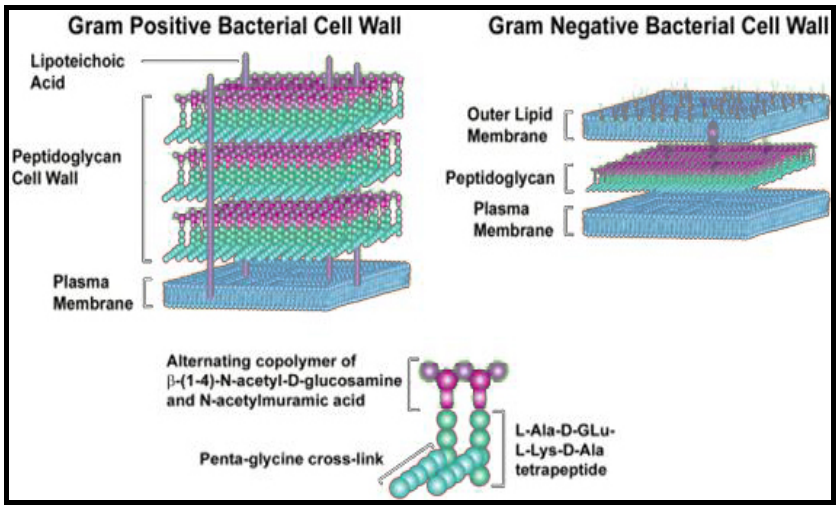

Figure (5): Illustration of the outer membrane, cell wall and plasma membrane of a Gram-positive & Gram-negative bacterium. Note: in Gram-positive, the wall is relatively thick and consists of many layers of peptidoglycan interspersed with teichoic acids. While in Gram-negative bacterium, the wall is relatively thin and contains much less peptidoglycan also, teichoic acids are absent. However, the Gram negative cell wall consists of an outer membrane that is outside of the peptidoglycan layer. The outer membrane is attached to the peptidoglycan sheet by a unique group of lipoprotein molecules (***Nikaido, 2003).***

Resistance to β-lactam antibiotics

Bacteria can avoid the bactericidal effect of β-lactams through three major ways (Figure 6):

A. Drug inactivation or modification through the production of β-lactamases ***(Bebrone et al., 2010).***

B. Alteration of target site: e.g. alteration of pencillin binding proteins (PBP), the binding target site of penicillins, in penicillin-resistant bacteria ***(Maree et al., 2007)*** **and** ***(Van de Velde et al., 2009).***

C. Reduced drug accumulation: by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface ***(Bohnert et al., 2010)***.

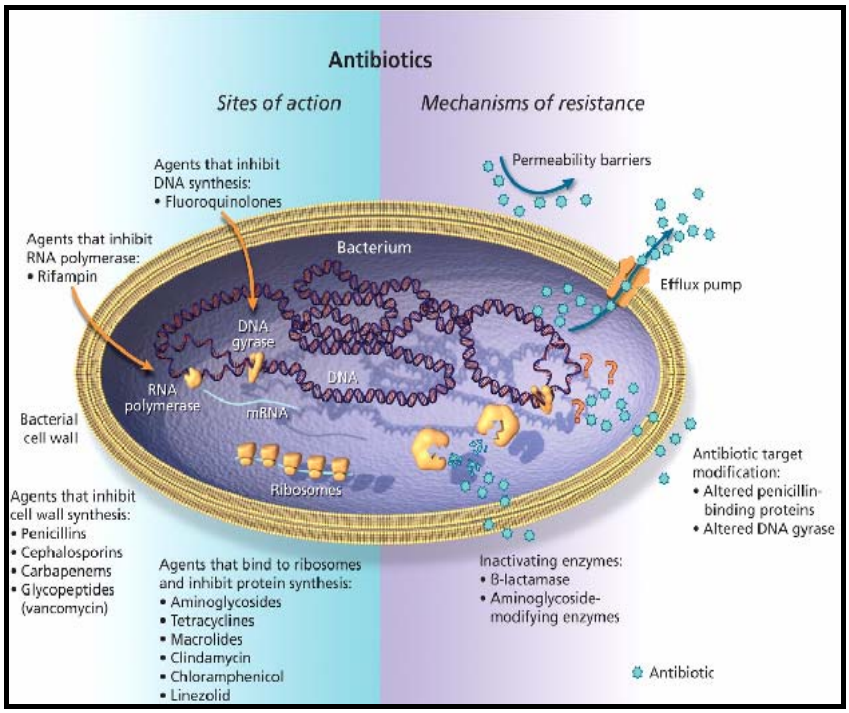

Figure (6): Sites of action and potential mechanisms of bacterial resistance to antimicrobial agents ***(Mulvey and Simor, 2009).***

β-lactamases

Beta-lactamases can be chromosome, plasmid, or transposon-expressed enzymes that are produced in a constitutive or inducible manner (Figure 7) ***(Livermore, 2009).*** These enzymes make biologically inactive products of the antibiotic by efficient hydrolysis of the amide bond in the β-lactam ring ***(Poole, 2004).***

β-Lactamases continue to evolve with CTX-M type extended-spectrum β-lactamases, AmpC, and carbapenamases as major threats for β-lactam therapy. *Enterobacteriaceae* often possess this type of mechanism in addition to drug efflux systems. Drug efflux pumps play a key role in drug resistance and also serve other functions in bacteria ***(Li and Nikaido, 2004).***

β-lactams diffuse through porin channels in the outer membrane to get access to their target; the PBPs. Mutations in the porin genes of Gram-negative bacteria confer a decrease in permeability in the outer membrane. Synergistic effects can occur with the expression of β-lactamases or an active efflux, making the bacteria even more resistant ***(Poole, 2004).***

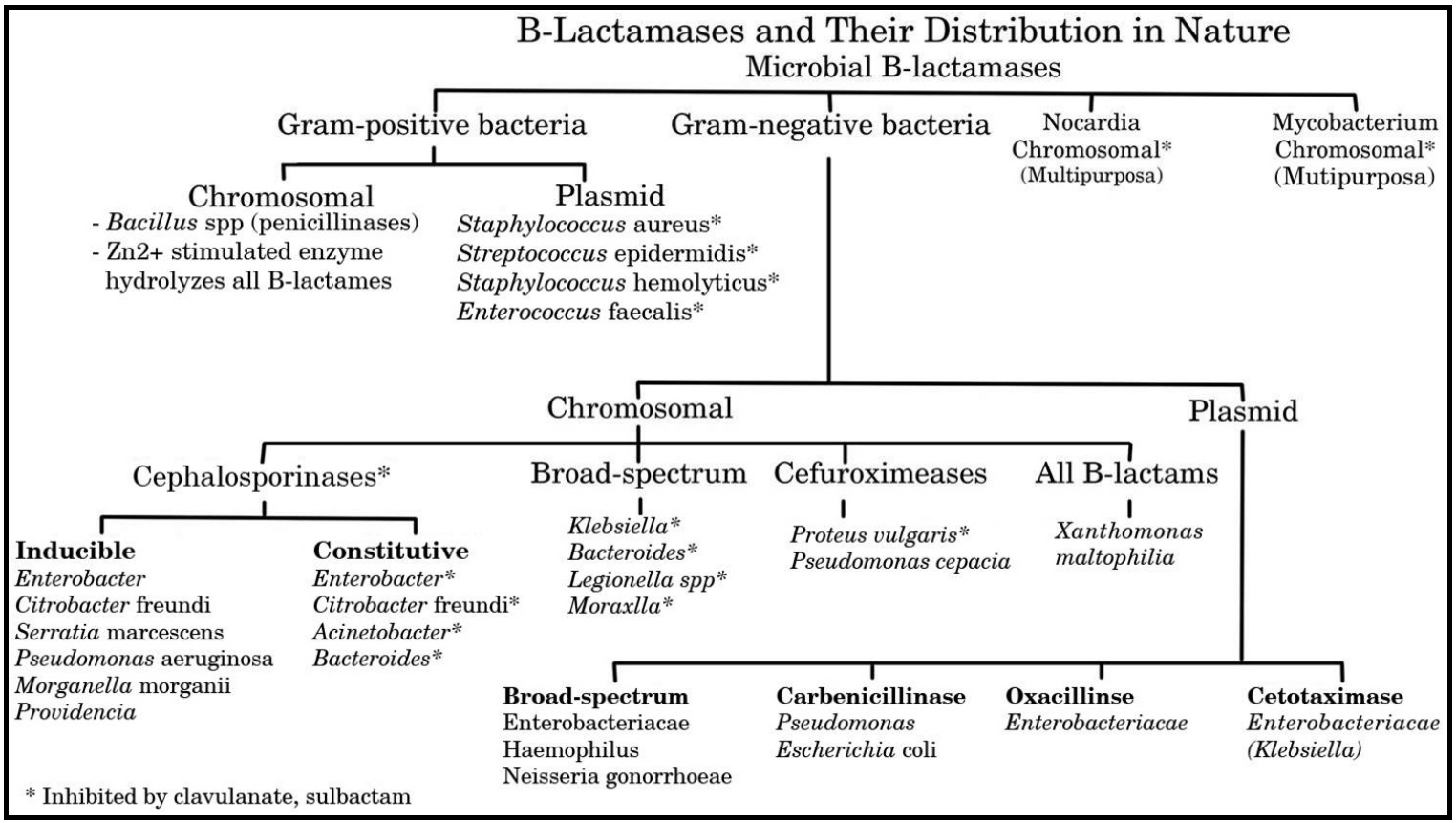

Figure (7): (-Lactamase found in bacteria and their classification and synthesis, whether chromosomally or plasmid mediated ***(Alice and John, 2004).***

Classification of β-Lactamases

Because of the diversity of enzymatic characteristics of the many β-lactamases discovered, multiple attempts have been made to categorize them since the late 1960s. These classifications involve two major approaches: the first and older one is based on the biochemical and functional characteristics of the enzyme, whereas the second approach is based on the molecular structure of the enzyme. In the former classification scheme, several criteria are used, including the spectrum of antimicrobial substrate profile, enzyme inhibition profile, hydrolysis rate (Vmax), binding affinity (Km), isoelectric focusing (pI), protein molecular weight, and amino acid composition. The molecular classification of β-lactamases is based on the nucleotide and amino acid sequences in these enzymes. To date, four classes are recognized (A-D), correlating with the functional classification. Classes A, C, and D act by a serine based mechanism, whereas class B or metallo β-lactamases need zinc for their action ***(Kong et al., 2010).*** The class B enzymes have been divided into three sub-classes B1, B2 and B3(Table1) ***(Singh et al., 2009).***

Briefly, class A enzymes are mostly plasmid-mediated penicillinases, such as those belonging to TEM and SHV subclasses. However, some of the evolved class A β-lactamases accept cephalosporins as substrates and are known as ESBLs, even though there are ESBL enzymes belonging to other classes as well.