CHAPTER 2

Literature Review

2.1. *Escherichia coli*

Escherichia coli (*E. coli*) is classified as part of Kingdom: Eubacteria, Phylum: Proteobacteria, Class: gamma-proteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae, Genus: *Escherichia. E. coli* is a Gram-negative, facultative anaerobic and non-spore forming bacteria and is commonly rod-shaped with 1.5-2 μm long and 0.5 μm wide (Figure 2.1). 37^{o} C is common growth conditions for *E. coli* but some laboratory strains can grow at temperature of up to 49^{o} C (Fotadar *et al*., 2005). Wild-type *E. coli* do not require special growth factors. *E. coli* is motile by peritrichous flagella (Darnton *et al*., 2007). The bacteria can grow both in the absence and presence of oxygen. Under anaerobic conditions, it can grow by fermentation pathways and -=adapt and live in either an anaerobic intestinal habitats or aerobic and anaerobic extra-intestinal habitats.

E. coli can respond to various environmental signals including temperature, chemical, osmolarity, and pH. For example, it can recognize the presence or absence of gases and chemicals in its environment and swim toward or away from them. It can use fimbriae to attach cell or surface receptor. In response to change in temperature and osmolarity, it can alter pore diameter of outer membrane porins to exclude inhibitory substances or import larger molecules.

E. coli is normal bacterial flora in the human intestinal tract. Most *E. coli* strains are harmless and produce necessary vitamin including vitamin K_2 (Bentley *et al*., 1982). However, some serotypes can cause serious food poisoning in humans (Vogt *et al*., 2002). The major route of pathogenic strains which cause disease is fecal-oral transmission. *E. coli* is able to survive in environment for a short time which makes them ideal indicator organism to test for fecal contamination.

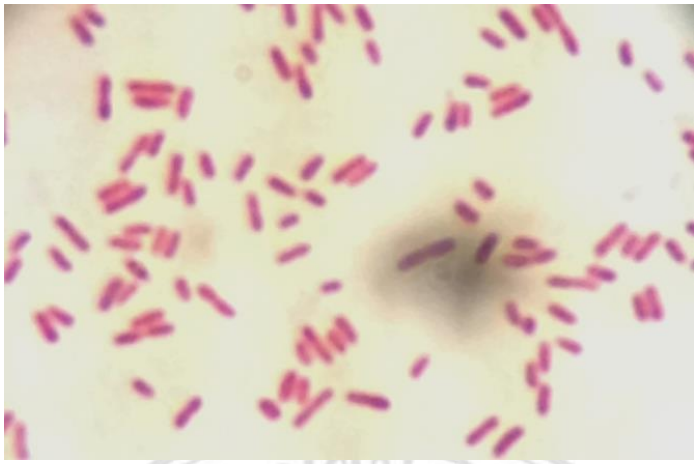

Figure 2.1 Gram stain of *E. coli*. *E.coli* is a Gram-negative bacilli by a standardized method of Gram stain interpretation.

2.2. Pathogenesis of *E. coli*

There are several highly adapted *E. coli* clones that have acquired virulence genes. Their virulences are frequently encoded on mobile genetic elements that can be horizontally transferred to other strains and create new combinations of virulence factors. Important virulence factors encoded by these genes comprise fimbrial adhesion, enterotoxins, cytotoxins, capsule and lipopolysacharide (LPS). Pathogenic *E. coli* may be differentiated by serotyping based on antigenic differences in the O antigen of LPS, flagellar or H antigen and capsule or K antigen (Orskov *et al*., 1977). In human, virulent strains of *E. coli* commonly cause three types of infiections, enteric or diarrhoeal disease, urinary tract infections (UTIs), and sepsis or meningitis.

2.2.1 Enteric or diarrhoeal disease

E. coli is well known for its ability to cause gastroenteritis or intestinal disease. There are six pathotypes of diarrhoeagenic *E. coli* (Figure 2.2) based on their interaction with target cell: enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC)

1) Enteroaggregative *E. coli* (EAEC) can aggregate tissue culture cell by their fimbriae. EAEC adheres to small and large intestine epithelial in a thick

biofilm and they produce heat stable (ST) enterotoxin and hemolysin similar to those of ETEC (Kaper *et al*., 2004). EAEC are non-invasive and cause only watery diarrhea without fever.

2) Enteropathogenic *E.coli* (EPEC) is important causative agent of diarrheal disease worldwide. The contact with an epithelial cell surface of the intestine, usually the colon, via bundle forming pili (BFP) can activate their type III secretion systems (Reis *et al*., 2010). Adherence to the intestinal mucosa causes a depolymerization of actin in the host cell, causing significant deformation leading to disruption of an electrolyte balance and final cell death. EPEC cells can induce an immune system response and are moderately invasive (Kaper *et al*., 2004).

3) Enteroinvasive *E. coli* (EIEC) is invasive. They can invade the colonic epithelial cell by penetration, lyses the endocytosis vacuole and pass through the cell by nucleating actin microfilaments. The clinical syndrome is dysentery-like diarrhea with fever identical to that from *Shigella* dysentery.

4) Enterohaemorrhagic *E. coli* (EHEC) is capable of producing shiga toxin and typically cause bloody diarrhea. The most important EHEC pathogens are O157:H7 serotype in the United Kingdom, North America and Japan whereas O26 and O111 serogroups have frequently been involved in sporadic cases and outbreaks (Kaper *et al*., 2004). The infection dose of these strains is very low, between 1 and 100 CFU. EHEC can cause both non-bloody and bloody diarrhea, hemolytic-uremic syndrome (HUS) and acute kidney failure. It uses *E. coli* common pilus (ECP) for attachment. It is moderately invasive and can produce shiga toxin that induce inflammatory response (Rendón *et al*., 2007).

5) Enterotoxigenic *E. coli* (ETEC) uses fimbrial adhesins to bind intestinal absorptive cell in the small intestine and secrete heat-labile (LT) and/or heat-stable (ST) enterotoxins that cause watery diarrhea. Though ETEC strains are noninvasive, they are the important bacterial cause of diarrhea in children in the developing countries, and the most common cause of traveler's diarrhea (Kaper *et al*., 2004).

6) Diffusely adherent *E.coli* (DAEC) have been considered a diarrheagenic group of *E. coli* (DEC). They are characterized by different pattern on cultured epithelial cell HeLa or HEp-2 cell monolayers (Servin AL, 2005). DAEC have been implicated as a cause of diarrhea in children > 12 months of age (Vogt *et al*., 2002). Fimbrial adhesion or a related adhesion is produced by approximately 75% of DAEC strains (Kaper *et al*., 2004). DAEC strains induce a cytopathic effect characterized by the development of long cellular extensions, which wrap around the adherent bacteria.

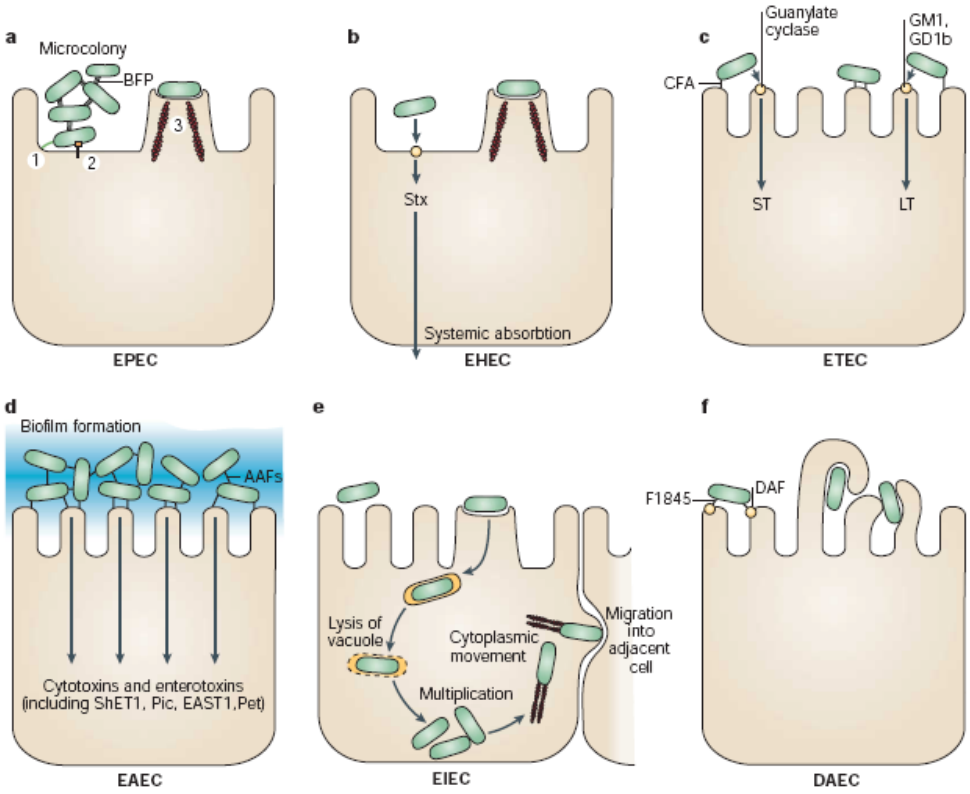

Figure 2.2 Pathogenic scheme of diarrhoeagenic *E. coli* (Kaper *et al*., 2004)

2.2.2 Urinary tract infection

Urinary tract infection (UTI) is a significant infection disease in human that cause morbidity and mortality with approximately 150 million worldwide per year. The primary cause of UTIs is uropathogenic *E. coli* (UPEC) (Totsika *et al*., 2012). UTIs are more prevalent in women. Female anatomy, family history and sexual intercourse are risk factors of the infections. Importantly, a small number of *E. coli* serogroup O are associated with acute pyelonephritis and cystitis in the

normal urinary tract (Kaper *et al*., 2004). The causative agent of pyelonephritis is the fecal bacteria that spread up pass urinary tract to the bladder and to the kidneys or the prostate in males. In pyelonephritis strains, type 1 fimbriae are lowly expressed by control of the invertible element (Gunther *et al*., 2001). The strain with few fimbriae are released from bladder epithelial cell receptor and spread upward to the kidneys, where the fimbriae attach specifically to the digalactoside receptors on kidney epithelium (Edén *et al*., 1987; Korhonen *et al*., 1986). At this stage, renal epitheliums are destroyed by haemolysin and LPS and acute inflammatory response recruits PMNs to the infection site. Moreover, secreted autotransporter toxin damages glomeruli and surrounding epithelium. On the other hand, type 1 fimbriae in strains that cause cystitis are highly expressed and the infection is restricted to the bladder (Connell *et al*., 1996).

2.2.3 Sepsis or meningitis

The most common cause of Gram-negative neonatal meningitis is *E. coli* pathotype with case fatality rate of 15-40% and severe neurological defects in many of the survivors. Most of *E. coli* pathogens carry known virulence factors, such as the capsule antigen K1 (Korhonen *et al*., 1985). Capsule can protect them from phagocytic cell and allows these bacteria through the blood-brain barrier because capsule contains sialic acid which is found in humans and does not induce innate immune system and plays a role in *E. coli* ability to penetrate pass barrier. Typically, the progression of neonatal meningitis starts with bacteria colonizes in lower gastrointestinal tract and then invades through into the blood which cause of sepsis. Finally, there are penetrating into CSF. The neonate's cannot defense against invading bacteria because their immune system are less effectively. The important cause early-onset meningitis in new born is the transmission from mother who has colonization of bacteria to the neonate.

2.3 Antibiotic therapy and resistance

The β-lactams are a group of antibiotics that have been used most intensively to medicate *E. coli* infections. This group includes amoxicillin, as well as other semi synthetic penicillins, many cephalosporin, carbapenems, aztreonam. Currently, third

generation cephalosporins are considered as the drugs of choice for treatment of infection caused by Enterobacteriaceae. Third generation cephalosporins or extended spectrum cephalosporins such as ceftazidime, ceftriaxone and cefotaxime received wide spread clinical use in the early 1980s in response to the spread of β-lactamase-producing strain in Enterobacteriaceae, especially TEM-1 and SHV-1 β-lactamases with ampicillin hydrolytic activities that spread among *E.coli* and *K.pneumoniae* and into *Hemophilus influenza* and *Neisseria gonorrhoeae*. The extended spectrum β-lactamases are effective against a wide range of microorganisms, particularly Gram-negative bacteria. They are very beneficial to the treatment of nosocomial infections.

Third generation cephalosporin are able to permeate the central nervous system (CNS), resulting them can be used to treat meningitis caused by *Pneumococci, Meningococci*, *H. influenzae* and susceptible *E. coli* (Prasad *et al*., 2007). Additionally, third generation cephalosporins had the major benefit of reduced nephrotoxin effects compared to aminoglycosides and polymyxin (Paterson and Bonomo, 2005). Third generation cephalosporins are bactericidal and have the same action of other β-lactams antibiotics such as penicillin. These antibiotics interrupt the synthesis of cell walls in susceptible microbes by interfering peptidoglycan layer synthesis. In the final transpeptidation step, penicillin binding proteins (PBPs) bind to D-Ala-D-Ala at the end of muropeptides and crosslink the peptidoglycan. β-lactam irreversibly inhibit crosslink of the peptidoglycan because their structural similarity to the D-Ala-D-Ala site of the muropeptide and, hence, bind to the target PBPs (Fisher *et al*., 2005). The development of many new β-lactams was aimed to increase resistance to the hydrolytic activity of β-lactamases. As soon new classes of β-lactams were developed, new β-lactamases have appeared and they have become less susceptible to the newly developed antibiotics. Presumably, the misuse and overuse of newly developed β-lactams were selective pressures for new variants of β-lactamases. Resistance genes are commonly associated with mobile genetic elements and can be transferred between distantly related bacteria (Wellington *et al*., 2013). *E. coli* frequently carry multiple antibiotic resistance genes on plasmids and transfer to other species. Class 1 integrons were found in 40 to 70% of gram negative pathogens and were common in *E. coli* isolates (van Essen-Zandbergen *et al*., 2007; Martinez-Freijo *et al*., 1998). They play important role in the worldwide

problem of multidrug-resistance gene. Because they can capture and incorporate resistance gene cassettes by site-specific recombinant and can express them (Domingues *et al*., 2012). They are often associated in various plasmids and transposons, facilitating their transfer into a wide range of pathogens (Gillings *et al*., 2008). *E. coli* are usually a reservoir of integrons-carrying isolates and can spread to other bacteria (Phongpaichi *et al*., 2008). Consequently, *E. coli* isolates are resistant to many classes of antibiotics. In addition, the most common frequent coresistances found in *E. coli* are ampicillin, tetracycline, streptomycin, choramphenical, aminoglycoides and sulphonamides as well as third generation cephalosporin (Hsu *et al*., 2006; Skurnik *et al*., 2003). Treatment of these multiple drug-resistant pathogens is a therapeutic challenge. Moreover, the biofilm setting allows *E. coli* to transfer plasmid to and accept plasmids from other bacteria (Salyers *et al*., 2004).

2.4 β-lactamase

The production of β-lactamase in Enterobacteriaceae is the most important mechanism of resistance to β-lactam antibiotic. The first β-lactamase was reported before the first β-lactam penicillin was used in medical practice (Abraham and Chain, 1940). β-lactamase enzymes showed some sequence homology with penicillin-binding proteins suggesting that β-lactamases might be evolved from these proteins. Ambler and colleagues classified β-lactamase enzymes into four classes, designated classes A, B, C, and D (Table 2.1), on the basis of their nucleotide and amino acid sequence similarity (Ambler *et al*., 1991). The most common classes are classes A and class C β-lactamases which confer resistance to the third generation cephalosporins. Class A, C and D β-lactamases are serine hydrolases. These serine-based enzymes react non-covalently with the β-lactam ring and attack by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Following protronation of the covalent complex and cleavage of the C-N bond by activated water molecule the attacks the covalent complex occur. The hydrolysis of the ester finally releases active enzyme and inactive β-lactam (Shah *et al*., 2004 and Drawz and Bonomo, 2010). This mechanism is employed by β-lactamase of molecular classes A, C and D whereas class B metallo β-lactamase use metal ion zinc at the active site to destroy the β-lactam ring (Livermore, 1995).

2.4.1 Extended-spectrum β-lactamase (ESBLs)

The name extended-spectrum β-lactamase (ESBL) was used to explain the broad-spectrum hydrolysis profile of this enzyme (Livermore, 2008). The ESBL is the member of Ambler class A and have a serine at the active site (Ambler, 1980) with molecular mass of aproximately 29 kDa (Medeiros *et al*., 1988). Class A β-lactamase includes staphylococcal penicillinases, broad spectrum β-lactamase (TEM-1, TEM-2, SHV-1) and extended-spectrum β-lactamase. Class A β-lactamase catalytic mechanism is divided into two stages, acylation and deacylation (Christensen *et al*., 1990). Initially, a proton is removed from the active site of enzyme (catalytic Ser-70 residue) (Chen *et al*., 1996). It is transferred to the side chain amide group of Lys-73 residue or transferred to water molecule that is coordinated by Ser-70, Glu-116, and Asn-170. Then the oxygen of Ser-70 attacks the carbonyl group and destroys the amide bond of the β-lactam ring. This cause contributes to inactivate the antibacterial properties. (Strynadka *et al*., 1992). Only the molecular classification scheme does not adequately differentiate the many types of class A. Therefore, Bush, Jacoby, and Medeiros created a classification scheme which uses the molecular structure and nucleotide sequence plus the biochemical properties of enzyme to place β-lactamase into functional group 2be (Table 2.1) (Bush *et al*., 1995). The letter "e" of 2be for extended spectrum of activity which these enzymes are initiated from group 2b or broad-spectrum β-lactamases TEM-1, TEM-2, and SHV-1 (Bush *et al*., 1995; Paterson and Bonomo, 2005). As few as one amino acid substitution around the active site of these broad spectrum β-lactamases cause changes in the enzymatic activity and allows them to hydrolyze to penicillins, extended-spectrum cephalosporin and aztreonam. However, ESBLs are not active against carbapenems (imipenem, ertapenem, and meropenem) or cephamycins (most stains expressing ESBL are susceptible to cefoxitin and cefotetan), and can be inhibited by β-lactamase inhibitors such as clavulanate, sulbactam, or tazobactam (Bradford, 2001). ESBLs are commonly plasmid mediated rather than chromosomally mediated and the resistance genes can be transferred to other species of Enterobacteriaceae (Shah *et al*., 2004). Most ESBL are derivatives of

TEM or SHV enzyme (Bush *et al*., 1995; Gniadkowski *et al*., 1998). These are now> 200 TEM-type β-lactamase and >140 SHV-type enzyme (http://www.lahey.org/ studies/webt.htm). TEM- and SHV-type ESBLs are most often found in *E. coli*, *K. pheumoniae* and other Enterobactericeae. Unlike most ESBL, OXA type β-lactamases have been found mainly in *Pseudomonas aeruginosa* and only rarely in Enterobacteriaceae (Bradford, 2001).

Table 2.1 Classification of β-lactamase: The Ambler scheme and the Bush-Medeiros Jacoby system (Perez *et al*., 2007).

Bush-Jacoby-Medeiros system	Major subgroups	Ambler system	Main attributes
Group1 Cephalosporinases		C (Cephalosporinases)	Usually chromosomal; resistance to all β-lactamase except carbapenems; not inhibited by clavulanate
Group 2 penicillinases (clavulanic acid suscpetible)	2a	A (serine β-lactamases)	Staphylococcal penicillinases
	2b	A	Broad-spectrum: TEM-1, TEM-2, SHV-1
	2be	A	Extended-spectrum: TEM-3-160, SHV-2-101
	2br	A	Inhibitor resistant TEM (IRT)
	2c	A	Carbenicillin-hydrolyzing
	2e	A	Cephalosporinases inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
	2d	D (oxacillin-hydrolyzing)	Cloxacillin-hydrolyzing (OXA)
Group 3 metallo-β-lactamase	3a	B (metallo-β-lactamases)	Zing-dependent carbapenemases
	3b	B	
	3c	B	
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

1) TEM-type of ESBLs

TEM-type ESBLs are derivative of TEM-1 and TEM-2. TEM-1 was first identified in 1965 in *E. coli* clinical isolate from a patient named Temoneira in Athens, Greece (Datta and Kontomichalou, 1965). TEM-1 was the most prevalent β-lactamase in Gram-negative bacteria. Most of ampicillin resistances in *E. coli* isolates were associated with this enzyme (Livermore, 1995). TEM-2 has a single amino acid that differs from TEM-1 but exhibits the same hydrolytic profile to TEM-1. Therefore, TEM-2 enzyme is not classified as ESBL (Jacoby and Medeiros, 1991; Paterson and Bonomo, 2005). TEM-3 was first reported in 1989 from *K. pneumonia* isolates in France (Sougakoff *et al*., 1988). TEM-3 has amino acid substitution in two positions yielding it different from TEM-2 (Rasmussen *et al*., 1993). The first TEM derived ESBL type is TEM-12 that was isolated from *Klebsiella oxytoca* in 1982 in Liverpool, England (Du Bois *et al*., 1995; Paterson and Bonomo, 2005). The mutations within the $bla_{\text{TEM-1}}$ structural gene have allowed the TEM-12 enzyme to expand the hydrolysis capabilities. Since that first report, more than 200 TEM types of ESBLs have been described (http:www.lahey.org/studies/temtable.htm.). The combinations of amino acid substitutions at some position of TEM-1 enzymes (such as Glu104Lys, Arg164Ser or His, Gly238Ser, Glu204Lys) are important for producing the ESBL phenotype (Figure 2.3) (Bradford, 2001). These indicate natural TEM variants in clinical strains were occurred by selective pressure from several β-lactams drug rather than selection with a single agent. (Blazquez *et al*., 2000). In recently, TEM enzymes were classified based on their chemical properties into four types; (1) Broad-Spectrum TEM-Type β-lactamases (TEM-1) this enzyme phenotype were characterized by resistance to penicillins and and first-generation cephalosporins, (2) Inhibitor-resistant TEM β-lactamases (IRT) have similar hydrolysis profile with type 1 but resistance to β-lactam-clavulanate combination and susceptible to cephalosporins (Salverda *et al*., 2010), (3) TEM-Type Extended-Spectrum β-Lactamases have hydrolysis spectra same to that of other clavulanate-inhibited Ambler class A ESBL (Robin *et al*., 2007) and (4) complex mutant TEM β-lactamase (CMT) include TEM variants phenotype were characterized by extended-spectrum of hydrolysis profile which they are not inhibited by β-lactamase inhibitors (Partridge, 2011).

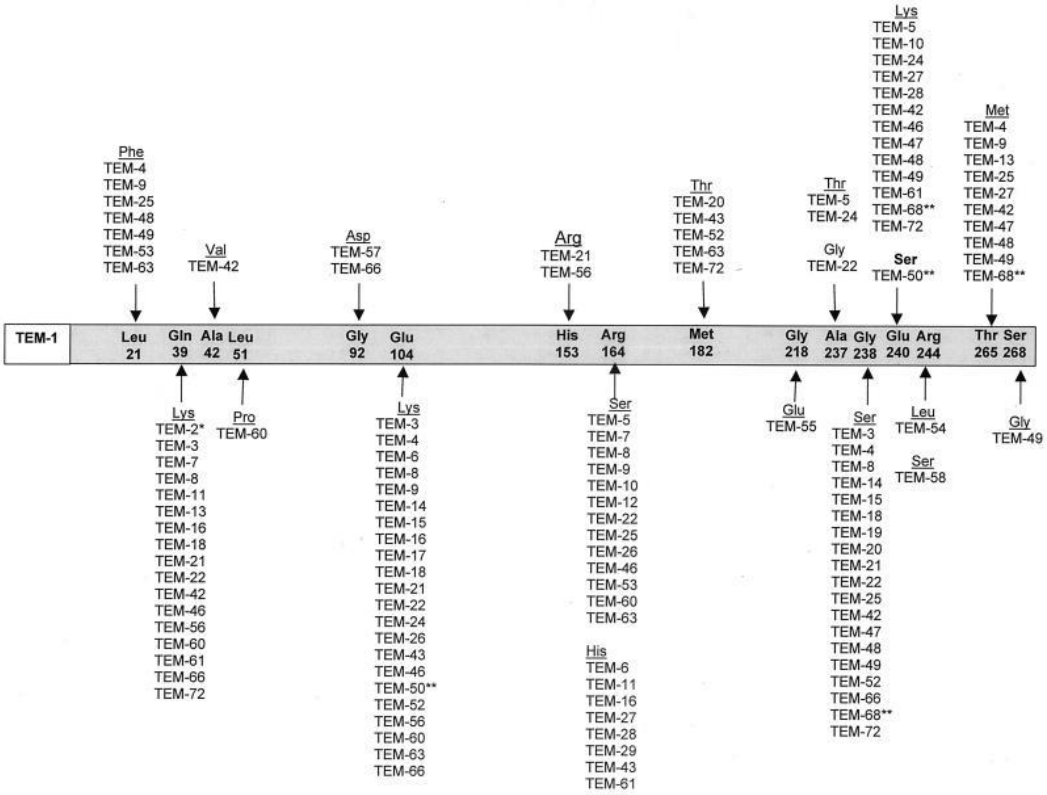

Figure 2.3 Amino acid substitutions in TEM ESBL derivatives. Amino acid substitutions at some position of TEM-1 enzymes (Bradford, 2001).

The closely related transposons identified as the carrier of *bla*$_{\text{TEM}}$ genes were primarily designated TnA (Hedges and Jacob, 1974). Recently, All variants of TEM-1 or TEM-2 are encoded by the bacterial resistance transposons to be idedtified, namely Tn1 (Hedges and Jacob, 1974), Tn2 (Heffron *et al*., 1975), Tn3 (Kopecko and Cohen, 1975) and Tn801, etc., which depend on the *bla*$_{\text{TEM}}$ variant. *bla*$_{\text{TEM}}$ ESBL genes such as *bla*$_{\text{TEM-3}}$, *bla*$_{\text{TEM-21}}$ and *bla*$_{\text{TEM-24}}$ usually mobilized by Tn*3*-like structure or Tn*3* transposons. They located on IncA/C plasmid, although *bla*$_{\text{TEM-1}}$ or Inhibitor-resistant TEM β-lactamases that were prevalent in *E. coli* commonly located on IncF plasmids and not located on IncA/C plasmid (Mabilat *et al*., 1992; Marcadé *et al.*, 2009). Moreover, *bla*$_{\text{TEM-1}}$ gene frequently coexists with blaCTX-M-15 gene on IncF plasmid, because the target site for the integration of IS*Ecp1-bla*$_{\text{CTX-M-15}}$ is Tn*3* transposons (Boyd *et al*., 2004; Carattoli, 2009).

2) SHV-type of ESBLs

The nomenclature “SHV” refers to sulfhydryl reagent variable because of the variable inhibition activity of SHV by *p*-chloromercuribenzoate (Sykes and Bush, 1982) (This activity was never confirmed in later studies with purified enzyme). SHV-type ESBLs are major found *in K.pheumonia* and other bacteria have also been found such as *E. coli*, *Citrobacter divesus*, and *P. aeruginosa* (Bradford *et al*., 1995; El Harrif-Heraud *et al*., 1997; Naas *et al*., 1999; Rasheed *et al*., 1997). SHV-type ESBLs are characterized by hydrolytic activity against penicillins and broad-spectrum cephalosporins. They are not against cephamycins and carbapenems (Poirel *et al*., 2012). The first SHV is SHV-1 identified by Petit and colleagues from genus *Klebsiella* which chromosomally encoded β-lactamase (Pitton, 1972). SHV-1 enzyme had narrow-spectrum of activity and hydrolyzed penicillin (Heritage *et al*., 1992). Many years later, first SHV-type ESBLs, SHV-2, was reported in clinical isolates of *K. pneumoniae, Klebsialla ozaenae* and *serratia marcescens* in 1983 (Knothe *et al*., 1983). SHV-2 had a single amino acid substitution at position 238 SHV-1 was replaced by serine in SHV-2 that change hydrolytic activity to encompass extended-spectrum cephalosporins (Barthélémy *et al*., 1988). The substitutions that occur in fewer positions in bla_{SHV} structural gene these changes create new SHV variants (Figure 2.4) (Bradford, 2001). For example, In the case of SHV-2 the amino acid substitutions occur at position 238 contribute to the cavity of enzyme is enlarged and lead to the bulky structure of drugs (such as extended-spectrum cephalosporins) enter the active site of β-lactamase enzyme (Huletsky *et al*., 1993). The outcome of this change is related with a higher affinity during hydrolysis. However, the majority of SHV-type derivatives have biochemical characteristics similar to the ESBL and some derivatives have an inhibitor-resistant phenotype (SHV-10). This enzyme derived from SHV-9 that was previously reported as SHV-5a and in SHV-10, serine was replaced by glycine at position 130 (Prinarakis *et al*., 1997).

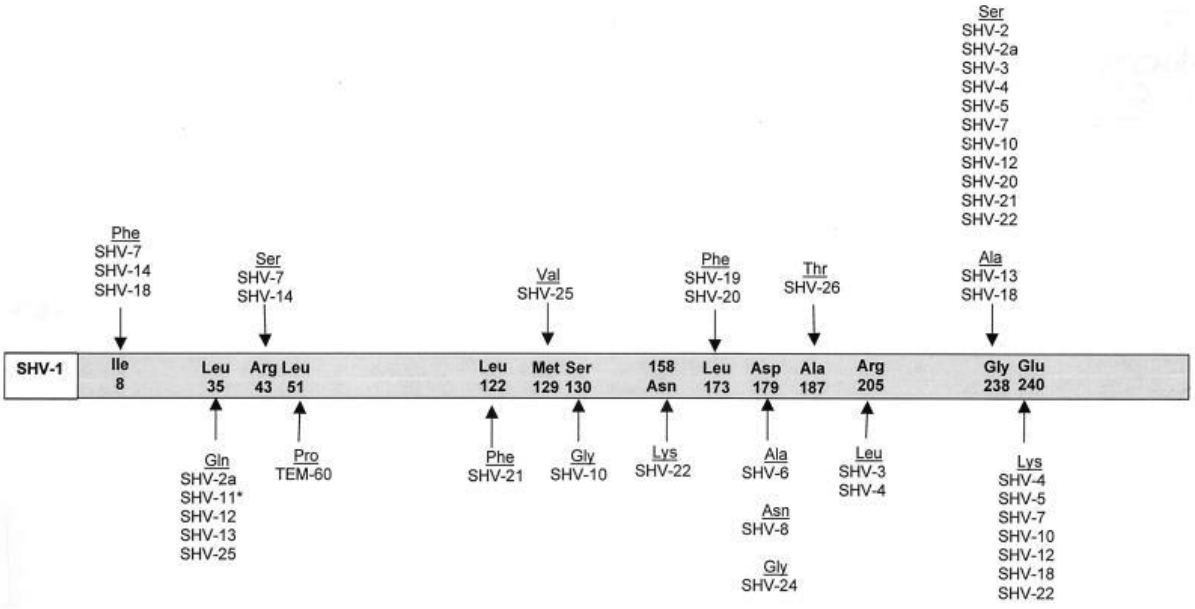

Figure 2.4 Amino acid substitutions in SHV ESBL derivatives (Bradford, 2001).

Genetic elements commonly found upstream of *bla*$_{SHV}$-like gene was transposable elements *IS*26 in several Enterobacterial isolates. This element is a key feature in acquirement of *bla*$_{SHV}$ genes and increases promoter strength (Podbielski *et al*., 1991). Moreover, the absence of either of the two IS*26* insertions that organism was susceptible to extended-spectrum cephalosporins (Hammond *et al*., 2008). Within 20 years of the discovery of this enzyme, SHV-2, SHV2a, SHV-5 and SHV-12 were the common type that spread around the world. *bla*$_{SHV-5}$ located on IncL/M plasmid and *bla*$_{SHV-12}$ located on Incl-1 plasmids (Carattoli, 2009).

3) CTX-M-type of ESBLs

In the past 10-15 years, new enzyme of class A ESBL which have 40% or less identity with TEM and SHV-type ESBL. This enzyme is CTX-M (Issack *et al*., 1995). The name CTX reflects the efficient hydrolytic activity of these β-lactamase resistances to cefotaxime. CTX-M-type enzymes confer the resistances to penicillins and extanded-spectrum cephalosporins with most variants are able hydrolyzed cefotaxime more than ceftazidime (Bonnet, 2004). This characteristic of CTX-M enzyme is different from TEM and SHV such as, TEM-3, TEM-4, SHV-2 and SHV-5) that has stronger activity against ceftazidime than against cefotaxime (Bonnet, 2004). Furthermore, this enzyme is higher rates of susceptibility to tazobactam (β-lactamase inhibitor) than to sulbactam and clavulanate (Bradford *et al*., 1997; Ma *et al*., 1998; Sabaté *et al*., 2000; Tzouvelekis *et al*., 2000).

In 1986, in Japan, the first emerge of CTX-M β-lactanase, non-TEM non-SHV ESBL in *E. coli* isolated from a laboratory dog (Matsumoto *et al*., 1988). It was used for pharmacokinetic studies of β-lactam antibiotic. This enzyme was designated as FEC-1 (Fecal *E. coli*) afterward it was related to CTX-M-3 which found in Poland (Bonnet, 2004). A few years later, the recognizing an ESBL from CTX-M-group on *E. coli* strain from ear exudates of a newborn in Munich, Germany in 1989. The strain was highly resistant to cefotaxime (MIC 128 mg/l) but ceftazidime susceptible, which designated CTX-M-1(Bauernfeind *et al*., 1990). In addition, the similar type of ESBL was reported in clinical *E. coli* strain MEN, isolated from an Italian patient in France in 1986. In the same year, this enzyme was sequenced and called MEN-1 (Barthélémy *et al*., 1992; Bernard et al., 1992). In 1996, this enzyme was found identical to CTX-M-1 (Bauernfeind *et al*., 1996). Previously, these enzymes were the most closely related (73 to 77% homology) to class A cephalosporinases encoded on the chromosome of *K. oxytoca, C. diversus, Proteus vulgaris*, and *Serratia fonticola* (Bauernfeind *et al*., 1996; Bonnet *et al*., 1999). However, recently these had a high homology with the chromosomal AmpC β-lactamase enzyme of *Kluyvera ascorbata*. It suggests that this species might be source of this enzyme. CTX-M group is plasmid-mediated. Phylogenetic study of the CTX-M family of β-lactamase revealed five major groups, subgroup 1, 2, 8, 9, 25, based on amino acid sequence similarities, (Figure 2.5)

(Bonnet, 2004) The members in the same group share >94% identity, whereas ≤ 90% identity is observed between the members belonging to distinct group. The CTX-M-1 subgroup includes enzyme of major clinical impact, especially CTX-M-15, CTX-M-3, and CTX-M-1, but also the CTX-M-10, CTX-M-11, CTX-M-12, CTX-M-22, CTX-M-23, CTX-M-28, CTX-M-30 and CTX-M-32 variants (Rossolini *et al*., 2008). Now, this group spreads worldwide includes Asia, Europe and North America (Livermore , 2007; Bush, 2008). The CTX-M-2 subgroup includes: CTX-M-2, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20, CTX-M-31, CTX-M-35, CTX-M-43, and CTX-M-44 variants. The CTX-M-8 subgroup includes the CTX-M-8, CTX-M-40 and CTX-M-63 variants. The CTX-M-9 subgroup includes the CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-18, CTX-M-19, CTX-M-21, CTX-M-24, CTX-M-27 and CTX-M-38 variants. And the CTX-M-25 subgroup includes the CTX-M-25, CTX-M-26, CTX-M-39, and CTX-M-41 variants (Rossolini *et al*., 2008). Mature CTX-M enzymes include 873 nucleotides corresponding to 291 amino acid residues, which is a protein with a molecular mass of 29 000 Da. (Ma *et al*., 1998). CTX-M types show variation in isoelectric points range from 7.4 to 9.1. This enzyme possesses the serine residue located at position 237. It plays an important role in the extended-spectrum hydrolysis profile (Tzouvelekis *et al*., 2000). Amino acid sequence of CTX-M enzymes correlated with natural β-lactamase of *Kluyvera* species. This species are environmental bacteria that are often isolates from water, soil, sewage. bla_{Klu} genes don't express the β-lactamase and are susceptible to inhibition by cefotaxime. These information demonstrated $bla_{\text{CTX-M}}$ genes originate from this bacteria. *Kluyvera georgiana* are the progenitors of $bla_{\text{CTX-M-8 subgroup}}$ (Poirel *et al*., 2002), and $bla_{\text{CTX-M-9 subgroup}}$. *Kluyvera ascorbata* and *Kluyvera cryocrescens* are progenitor of $bla_{\text{CTX-M-1 subgroup}}$ and $bla_{\text{CTX-M-2 subgroup}}$ (Bonnet, 2004).

Mobilization of $bla_{\text{CTX-M}}$ genes have been found to be involved with insertion sequences located upstream such as IS*Ecp1*, IS*CR1*, IS*26* IS*10*, and in rare cases phage-related elements (Poire *et al*., 2003; Oliver *et al*., 2005). IS*Ecp1* associated with all subgroups (CTX-M-1, CTX-M-2 and CTX-M9 subgroup) except CTX-M-8. Moreover, IS*CR1* found to be link to several members of CTX-M-2 and CTX-M9 subgroup and IS*10* with CTX-M-8. In addition, phage-related elements only related to $bla_{\text{CTX-M-10}}$ gene in Spain (Oliver *et al*., 2005). The P_{out} promoter of insertion sequence

can promote the expression and have a role in the selection and dissemination *bla*$_{CTX-M}$ gene. This information can describe the rapid worldwide spread of CTX-M-type enzymes (Poirel *et al*., 2003). Furthermore, a single copy of IS*Ecp1* is enough to mobilize the gene from chromosome of *Kluyvera* strain (Lartigue *et al*., 2006). ESBL *bla*$_{CTX-M}$ genes were associated with narrow (IncF group, InHI2 and IncI) and broad host-range (IncN, IncL/M, IncP-1-α and IncA/C) plasmid with variable transfer frequencies (10^{-7} to 10^{-2} per donor cell) (Canton and Coque, 2006).

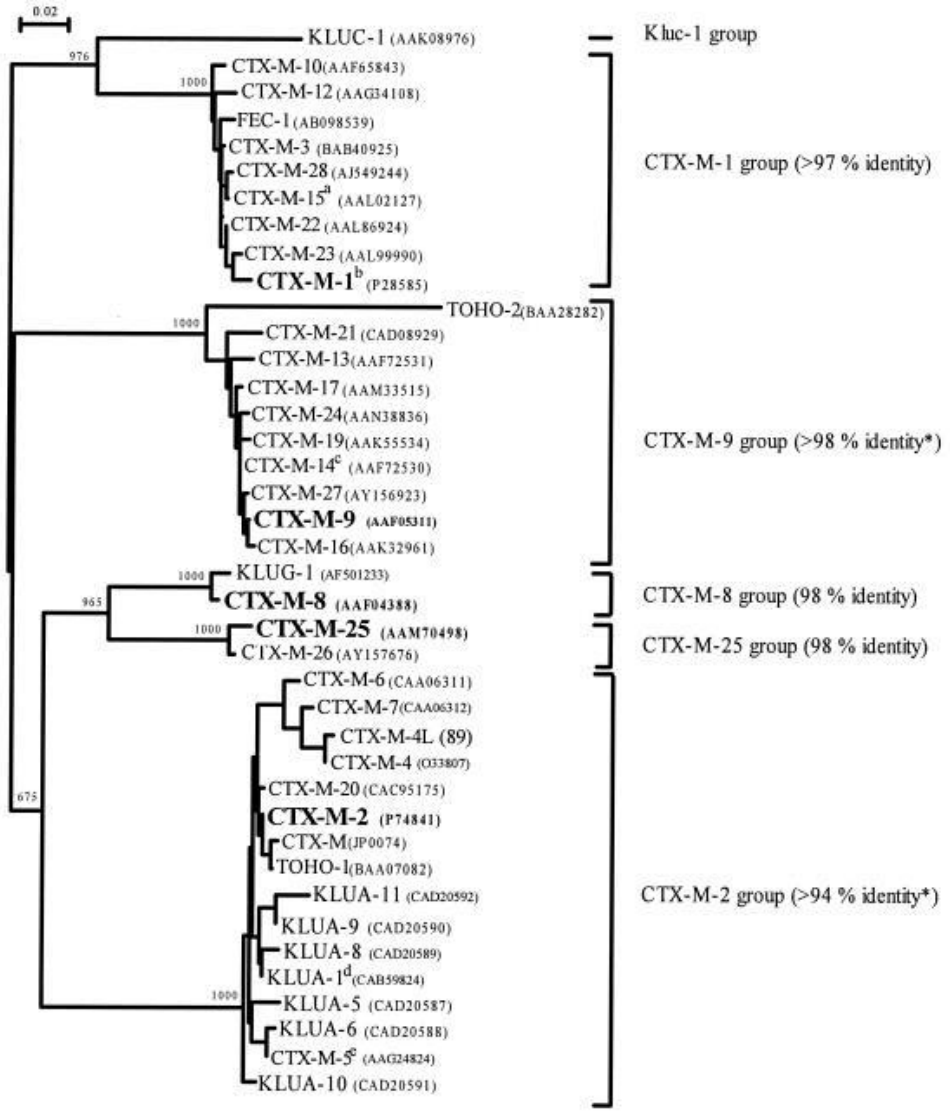

Figure 2.5 Dendrogram of CTX-M families (Bonnet, 2004).

2.4.2 AmpC β-lactamase

Cephalosporinases are the original name of Class C β-lactamases that referred to hydrolytic activity against cephalosporins. This class is the second most important families of β-lactam antibiotics. AmpC β-lactamse (class C) is not ESBL (class A) by the standard classification. Nevertheless, the AmpC enzymes correlate with ESBLs enzyme because the phenotypes of the 2 class overlap. Class A and C enzyme have ability to hydrolyse penicillins and cephalosporins. AmpC β-lactamase are less inhibited by class A β-lactamase inhibitor, while some are inhibited by tazobactam or sulbactam (Bush *et al*., 1993; Kazmierczak *et al*., 1990; Monnaie and Frere, 1993).

In 1965 Eriksson-Grennberg and Swedish investigators began a systemic study of the "Resistance of *E. coli* to penicillins". They mutated genes with stepwise and evaluate the rate of resistant to penicillin. Those genes were designated *ampA* and *ampB* (Eriksson-Greenberg, 1968; Eriksson-Greenberg *et al*., 1965). In *E. coli* K-12 strain reduced the penicillinase activity due to the mutation in the *ampA* gene and then was designated *ampC*. There data suggest that *ampA* was a regulatory gene for the penicillinase (Linström *et al*., 1970). Afterwards, in 1981, 1,536-nucleotide-long sequence of the *ampC* gene from *E. coli* had been determined. (Jaurin and Grundström, 1981). The sequence of this enzyme differed from other penicillinase-type β-lactamse but, they had similarly serine at them active site (Knott-Hunziker *et al*., 1982). In many Enterobacteriaceae carries an inducible AmpC cephalosporinase which chromosomally encoded AmpC β-lactamase. The transfer of inducible chromosomal AmpC β-lactamase genes to plasmids that is most without induction capabilities. Plasmid was sent to other organisms which express or do not express this type of β-lactamase such as *Klebsiella* app., *Salmonella* spp. or *E. coli*.

The enzyme, called CMY-1 that have cephamycinase activity profile and was more sensitive to sulbactam (β-lactamase inhibitor) than to tazobactam or clavulanate, suggesting that it might be a class C enzymes. However, the first reported unequivocal demonstration of a plasmid-mediated AmpC β-lactamase by Papanicolaou and colleagues, in 1990 (Papanicolaou *et al*., 1990). They reported β-lactamse gene named MIR-1 which confers resistance to α-methoxy- and oxyimino-β-lactams. This enzyme was similar to ampC gene of Enterobacter cloacae at 90% sequence identity

(Papanicolaou *et al*., 1990). Subsequence, plasmid-mediated class C β-lactamase has been discovered around the world. They was designate according to the to the site of discovery, such as the Dhahran hospital in Saudi Arabia (DHA) or Miriam hospital in Providence, Rhode Island. (MIR-1), to the resistance produced to cephamucins (CMY), cefoxitin (FOX), and moxalactam (MOX) or latamoxef (LAT), and to the type of β-lactamase, such as AmpC type (ACT) or Ambler class C (ACC). The *ampC* genes were located on plasmids of sizes varying from 7 to 180 kb (Horii *et al*., 1994; Stapleton *et al*., 1999). Plasmid was non self-transferable but was transferable by transformation or mobilization (Gazouli *et al*., 1996; Gazouli *et al*., 1998; Stapleton *et al*., 1999; Wu *et al*., 1999). Plasmid-mediated AmpC-type β-lactamase has isoelectric points between 6.4 and 9.4. The evident molecular masses of the mature plasmid-mediated AmpC β-lactamase vary from 38 to 42 The three-dimensional (3D) structures of AmpC enzyme include α-helical domain and α/β domain. The center of structures is the active site that has motif SXXK (X is any amino acid) at position 64 to 67 (Jacoby, 2009). The positions 315 to 317 that include Lys-Ser/Thr-Gly motif are significant role in forming the tertiary structure of the active site. In addition, Typ150 forms part the class C typical motif Typ-X-Asn is also important (but not essential) for catalysis of β-lactam hydrolysis (Dubus *et al*., 1994; Oefner *et al*., 1990).

Plasmid-mediated AmpC enzymes have been reported around the world but are less common than ESBLs. AmpC enzyme usually found in *K. pneumonaiae* and in other bacterial species not naturally producing *AmpC* enzymes such as *K. oxytoca*, *Salmonella* and *P. mirabilis*. In *E. coli*, case of cefoxitin resistance from Plasmid-mediated AmpC enzymes less than the overproduction of chromosomal AmpC β-lactamase (Jacoby, 2009).There enzyme have been classified into nine groups, including 90 variants of CMY, 13 variants of ACT and 10 of FOX, 8 variants of DHA and MOX, 5 of MIR and ACC and CFE-1 and LAT-1 (http://www.lahey.org/Studies/) (Jacoby, 2009). CMY-2 is the most prevalent of the plasmid-mediated AmpC enzymes and widely geographic spread. Most other strains producing this enzyme have been isolated from patients after several days in hospital and most common treated with cefoxitin, cefotetan, cefmetazole, imipenem or moxalactam (Bradford *et al*., 1997; Gonzalez Leiza *et al*., 1994; Horii *et al*., 1994 Papanicolaou *et al*., 1990). *bla*$_{\text{CMY-2}}$ gene

were associated upstream with a specific transposon-like element (IS*Ecp1*) and downstream with *blc* (gene of lipoprotein) and *sugE* (gene of multidrug efflux system protein) (Kang *et al*., 2006; Su *et al*., 2006). Insertion sequence IS*Ecp1* that mobilize the downstream-located genes and provides a strong promoter sequence for overexpression of β-lactamase enzymes. In addition, the strains with a plasmid-mediated AmpC emzyme also produced other β-lactamase such as TEM-1, TEM-2 or even an ESBL, such as SHV-5 (Gazouli *et al*., 1996; Gazouli *et al*., 1998; M'Zali *et al*., 1997).