CHARACTERISATION OF ANTIBIOTIC RESISTANCE MECHANISMS IN GRAM-NEGATIVE BACTERIA FROM TRIPOLI AND BENGHAZI, LIBYA

By

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Summary

As very little information is known of the antibiotic resistance in Gram-negative bacteria in Libya in addition to the desperate need for insight knowledge of the antibiotic resistance in Libyan hospitals, this study was undertaken to investigate the mechanism of antibiotic resistance in isolates collected from clinical, non-clinical and environmental samples from Tripoli and Benghazi, Libya. Bacterial collection include samples taken from patients admitted to the hospitals in ICUs and other wards, they also include swabs randomly collected from hospitals environment. These swabs were from walls, bedsides, curtains, floors, toilets, workstations, mechanical ventilators, stainless steel containers and instruments used in particular ICUs. This study clearly demonstrates the emergence of MDR Gram-negative bacteria in Tripoli and Benghazi hospitals, these MDR bacteria were clinical and non-clinical revealing the long standing infection control problem in these hospitals. *K. pneumoniae* was found as the most frequently isolated strain being disseminated in hospitals and outside hospitals followed by *E. coli*. *K. pneumoniae* and *E. coli* were detected harbouring $bla_{\text{CTX-M}}$ group1 in association with IS*Ecp1* the enhancer of the β-lactamase gene movement. More importantly, $bla_{\text{CTX-M-15}}$ in association with IS*Ecp1* were detected carried on conjugative plasmids of different sizes and able to move via Libyan *K. pneumoniae* and *E. coli* to sensitive bacteria via conjugation. Some isolates of *K. pneumoniae* were clonally related and were in some cases found in

different hospital revealing the outbreak of MDR *K. pneumoniae* in Libyan hospitals. *E.* coli strains showed the emergence of more than one clone in one hospital which indicates to the lack of hospital hygiene. Three novel sequence types among *K. pneumoniae* were discovered in this study, one of which *K. pneumoniae* AES817 that assigned ST511 was collected from one of Benghazi streets and was found carrying $bla_{\text{CTX-M-15}}$ and IS*Ecp1* on a plasmid of 400kb. Characterisation of *P. aeruginosa* showed the emergence of clonally related strains carrying $bla_{\text{VIM-2}}$, one was isolated from a patient admitted to Al-Jalla hospital in Benghazi and the other from a stainless steel container from the same hospital but different ward, this MBL was found on a novel integron in both strains. Interestingly, $bla_{\text{VIM-2}}$ was found chromosomally mediated proposing that the dissemination of this MBL might be due to mobile genetic elements. Perhaps the most interesting finding of this study is $bla_{\text{TMB-1}}$ which was detected in environmental strain swabbed from the floor of Tripoli central hospital. This MBL was unusual in terms of the similarity this gene shares with other known MBLs and also to the discovery of this MBL carried by environmental bacteria *A. xylosoxidans*, it is moreover the first MBL discovered in Libya.

Presentations and Publications

Presentations given from this study

1- Phenotypic and Genotypic Characterisation of Clinical and non-Clinical Gram-negative Bacteria from Benghazi-Libya. 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 12-15, 2009, San Francisco.

2- Identification of Tn*402*, Class 1 integrons and *ISCR* elements among endemic multi-drug-resistant *Klebsiella pneumoniae* from Benghazi-Libya. 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 12-15, 2009, San Francisco.

3- Novel subclass of a Group B1 Metallo-β-lactamase, bla_{TMB-1}, in Clinical and non Clinical Gram-negative Bacteria from Libya. 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 12-15, 2009, San Francisco.

4- The tniC-like transposon Tn*5090* is commonly found in *Klebsiella pneumoniae* isolates from Portugal and North Africa. 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 12-15, 2009, San Francisco.

Publications and publications in collaborations

1- Salabi, A. E., M. A. Toleman, J. Weeks, T. Bruderer, R. Frei, and T. R. Walsh. 2010. First report of the metallo-beta-lactamase SPM-1 in Europe. Antimicrob Agents Chemother 54:582.

2- Chouchani, C., R. Marrakchi, and A. El Salabi. 2011. Evolution of beta-lactams resistance in Gram-negative bacteria in Tunisia. Crit Rev Microbiol 37:167-177.

3- Chouchani, C., R. Marrakchi, L. Ferchichi, A. El Salabi, and T. R. Walsh. 2011. VIM and IMP metallo-beta-lactamases and other extended-spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* from environmental samples in a Tunisian hospital. Apmis 119:725-732.

4- Chouchani, C., A. El Salabi, R. Marrakchi, L. Ferchichi, and T. R. Walsh.Characterization of IncA/C conjugative plasmid harbouring bla_{TEM-52} and $bla_{CTX-M-15}$ extended-spectrum β-lactamases in clinical isolates of Escherichia coli in Tunisia (accepted).

5- Allaaeddin El Salabi, Pardha Saradhi Borra, Mark A. Toleman , Ørjan Samuelsen and Timothy R. Walsh Genetic and biochemical characterization of a novel metallo-β-lactamase, TMB-1, from a *Achromobacter xylosoxidans* strain isolated from Tripoli, Libya (submitted)

6- Allaaeddin El Salabi, Mark A. Toleman, Ahmed Matmati, Chedly Chouchani and Timothy R. Walsh bla_{VIM-2} positive *Pseudomonas aeruginosa* isolated from operating apparatus and patients in Tripoli, Libya (submitted)

7- Allaaeddin El Salabi, Mark A. Toleman, Abdulazizi Zorgani and Timothy R. Walsh. Molecular characterization of antibiotic resistance mechanisms in *K. pneumoniae* isolated from Tripoli and Benghazi hospitals (*in progress*)

8- Allaaeddin El Salabi, Mark A. Toleman, Asma Alramli and Timothy R. Walsh. Molecular characterization of antibiotic resistance mechanisms in *E. coli* collected from Tripoli and Benghazi hospitals (*in progress*)

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This thesis is dedicated to

the spirit of my father, the

spirit of my brother and

to the new Libya

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
AES	Allaaeddin El Salabi
AIM-1	Australian imipenemase
AmpC	ampicillin
ASP	Asparagin
attC	attachment site
bla	β-lactamase
CAI	community acquired infections
CIAI	complicated intra-abdominal infection
CR	common region
CSSSI	complicated skin and skin structure infection
CTX-M	Cefotaximase
CUTI	complicated urinary tract infection
CVL	Cervicovaginal Lavage
Cys	Cystein
Dhfr	dihydrofolate reductase
DIM	Dutch imipenemase
EARSS	European antimicrobial resistance surveillance system
EDTA	Ethylenediaminetetraacetic acid
ESBL	extended spectrum beta-lactamase

GFP	green fluorescent protein
GIM-1	Germany imipenemase
HAI	hospital-acquired infections
HIS	Histidine
IAI	intra-abdominal infection
ICARE	intensive care antimicrobial resistance epidemiology
ICE	Integration and conjugative element
ICU	intensive care unit
IMP	imipenemase
Int1	integrase gene
IPTG	Isopropyl-β-D-thiogalactoside
IS	insertion sequence
ISCR	insertion sequence common region
Kcat	catalytic rate constant
KHM-1	Kyorin Health MBL
Km	The Michaelis constant
KPC	Klebsiella pneumoniae carbapenemase
LPS	Lipopolysaccharide
MATE	multi-drug and toxic compound extrusion family
MBL	Metallo-β-lactamase
MDR	multi-Drug Resistant
MFS	major facilitator superfamily

MIC	minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration that kills 50% of the bacteria
MIC_{90}	Minimum inhibitory concentration that kills 90% of the bacteria
MDR	Multi-drug resistance
MLST	multilocus Sequence Typing
MYSTIC	meropenem yearly susceptibility test information collection
NDM-1	New-Delhi metallo-β-lactamase
NI	nosocomial infections
NP	nosocomial pneumoniae
OM	Outer membrane
OMP	outer membrane protein
ORF	open reading frame
OXA	oxacillinases
PBP	penicillin binding protein
PFGE	pulsed field gel electrophoresis
qacΔE1	quaternary ammonium compound
RAPD	Random amplified polymorphic DNA
RCS	recombination crossover site
RNA	ribonucleic acid
RND	resistance nodulation division

SHV	sulfhydryl variable
SDS	Sodium dodecyl sulphate
SIM-1	Seoul imipenemase
SLV	single locus varian
SMR	small multi-drug resistance
SPM-1	Sao Paolo metallo-β-lactamase
SSTI	skin and soft tissue infection
sull	sulphonamide resistance gene
SXT	trimethoprim sulphamethoxazole
TEM	Temoneira
TMB-1	Tripoli metallo-β-lactamase
tniC	transposase gene
US	united states
UTI	urinary tract infection
VAP	ventilator associated pneumonia
VIM	Verona Imipenemase

CONTENTS

Chapter Eight

Chapter One
General Introduction

1.1 Antibiotics

1.1.1 Introduction

Selman Waksman was one of the most recognized investigators in the field of bacteriology in 1940's, Waksman defined the term "antibiotic" as the substance that has the ability to kill bacteria (Bush, 2010a; Waksman & Woodruff, 1942). The term was singularly used to refer to a molecule that was bacteriostatic or bactericidal; however, today, the definition has changed and expanded - it is applied to natural products and synthetic chemicals that have antibacterial and antifungal activities. (Bush, 2010).

1.1.2 History of antibiotics

Antibiotics were introduced in the 1930's as a result of the discovery of the antibiotic penicillin from the fungus *Penicillium notatum* by Alexander Fleming in 1928 and the prontosil (sulfonamidochrysoidine) discovered by Gerhard Domagk in 1932. Such discoveries had a profound impact on human health and provided rapid and effective treatment of patients suffering from bacterial infections known to have been fatal. (Butler & Cooper, 2011).

β-lactam antibiotics were introduced clinically in 1940s exemplified by the antibiotic penicillin to treat bacterial infections caused by human pathogenic bacteria after approval of Food and Drug Administration (FDA) as before this time of the antibiotic era, infections such as bacteraemia caused by *Streptococcus pneumoniae* were the causative agents of mortality (Coates *et al.*, 2002; Dineen *et al.*, 1976). The introduction of antimicrobial agents helped

to decrease the mortality rates, e.g. the subcutaneous use of sulfanilamide caused reduction of acute meningococcal meningitis from 70-90% to nearly 10% (Powers, 2004). Between the 1930s and 1960s, more than 20 new classes of antibiotics were discovered – mainly natural or semi-synthetic (Table 1.1). As a result of these antibiotics to treat severe and life-threatening infections, the story has become a successful one (Butler & Buss, 2006; Powers, 2004) and has led to an over confidence on the ability of antimicrobials to eradicate all infectious diseases.

After the 1960s, research for new and novel drugs slowed and pharmaceutical industry paid less attention to antimicrobial research (Boucher *et al.*, 2009). This in part can be explained by the difficulty in discovering new antibacterial agents with completely novel mechanisms of action and also the cost of research – particularly clinical trials. (Coates *et al.*, 2002; Powers, 2004).

Since the intensive work on antimicrobial agents in the 20th century, only two new classes of antibiotics; daptomycin (Figure 1.1) and oxazolidinones (Figure 1.2) have recently been utilised to treat Gram-positive infections, whereas, innovation to address Gram-negative bacteria is still struggling and, at best, can only rely on modification of existing drugs e.g. fluoroquinolones (Figure 1.3), aminoglycosides (Figure 1.4), tetracyclines (Figure 1.5) and β-lactams. (Figure 1.6) (Bush & Pucci, 2011).

1.2 Gram-Negative Bacteria

Gram-negative bacteria are micro-organisms that are known to have an outer "cell envelope" or outer membrane (OM), which differs considerably from other bacterial strains in terms of structure and function. This "cell envelope" is composed of three envelope layers; the OM layer, the periplasm and the inner membrane or cytoplasmic membrane (Figure 1.7) (Gupta, 2011).

The structure of the OM has a unique lipid bilayer and its layers of phospholipids are confined to the inner side of the OM (Silhavy *et al.*, 2010). Glycolipids are main components of the OM and they are principally lipopolysacharides (LPS) which are located as an outer leaflet of the Gram-negative OM and play an important role as a functional barrier. LPS comprises the core of polysaccharide, lipid A, and extended polysaccharide chain O antigen. Lipid A is also known as the endotoxin, minute amounts of which can cause fever and septic shock syndrome. (Ryan *et al.*, 2004; Silhavy *et al.*, 2010).

The OM contains proteins which differ to proteins of the cytoplasmic membrane. Those proteins are classified into two groups; lipoproteins and β-barrel proteins. The function of most lipoproteins are not known yet, whereas the β-barrel proteins are known as Outer Membrane Proteins (OMPs) and have different roles according to the kind of OMP, for instance the function of

OmpF and OmpC are known as porins in *E. coli* allows the passive diffusion and facilitated movement of monosaccharides, disaccharides and amino

Table 1.1 History of antibiotic introductions and approval (according to Powers, 2004)

Antibiotic	Year of Discovery
Sulfonamides	1935 (launched)
B-lactams	1941 (launched)
Aminoglycosides	1944 (introduced)
Streptomycin	1947 (launched)
Chloramphenicol	1949 (launched)
Tetracycline	1950 (launched)
Macrolides	1952 (introduced)
Glycopeptides	1956 (introduced)
Rifamycins	1957 (introduced)
Nitromidiazoles	1959 (introduced)
Quinolones	1962 (introduced)
Nalidixic acid	1964 (introduced)
Gentamicin	1967 (launched)
Trimethoprim	1968 (launched)
Oxazolidinones	2000 (launched)
Linezolid	2000 (launched)
Lipopeptides	2003 (launched)

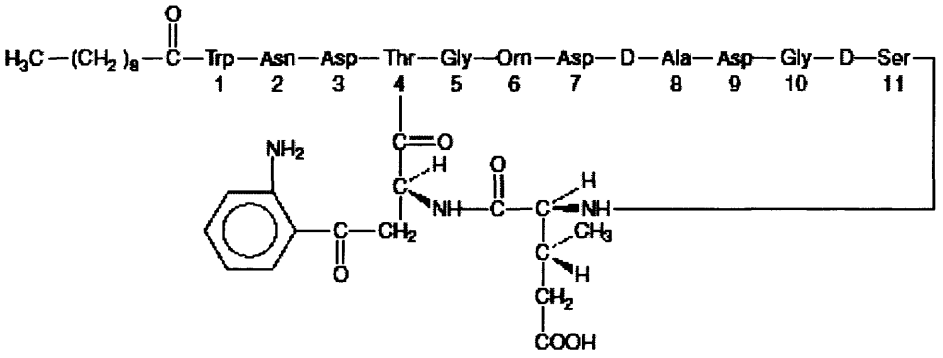

Figure 1.1 Chemical structure of Daptomycin (according to Bush & Pucci, 2011)

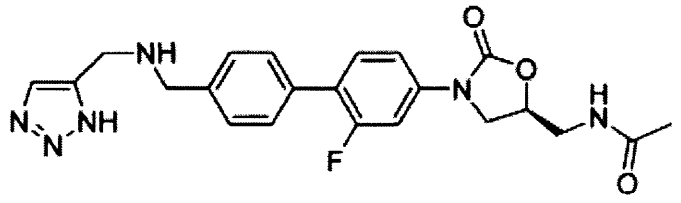

Figure 1.2 Chemical structure of the oxazolidinone radezolid (according to Bush & Pucci, 2011)

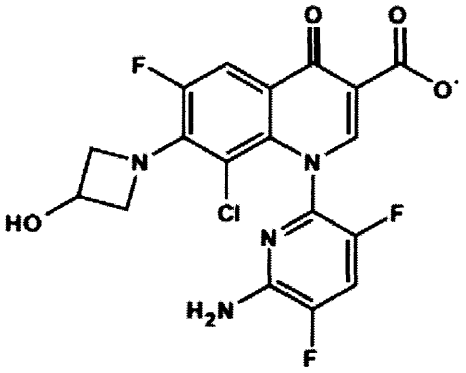

Figure 1.3 Chemical structure of the fluoroquinolone delafloxacin (according to Bush & Pucci, 2011)

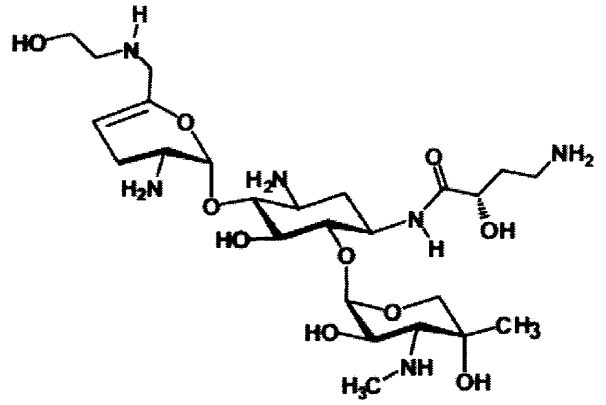

Figure 1.4 Chemical structure of the aminoglycoside ACHN-490 (according to Bush & Pucci, 2011)

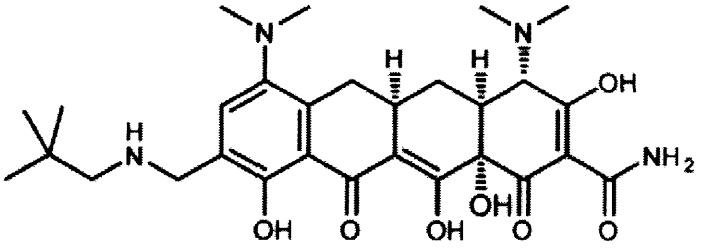

Figure 1.5 Chemical structure of the tetracycline omadacycline (according to Bush & Pucci, 2011)

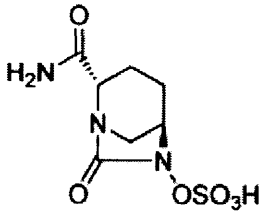

Figure 1.6 Chemical structure of the Avibactam NXL-104 (according to Bush & Pucci, 2011)

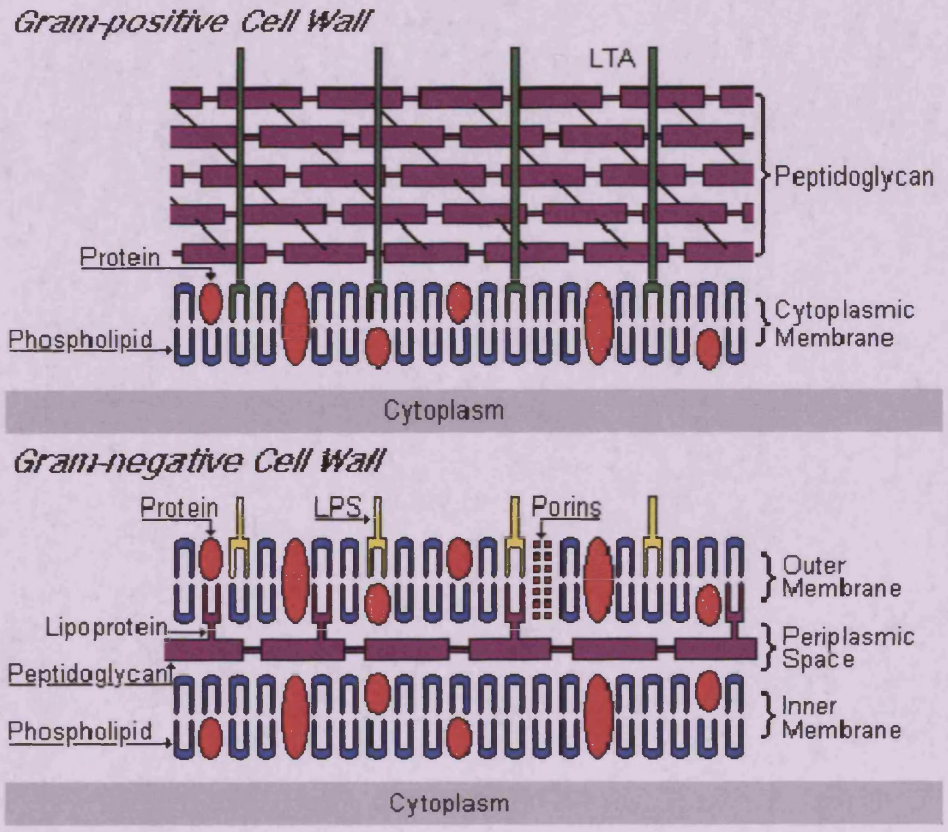

Figure 1.7 Cell wall envelopes of Gram-positive and Gram-negative bacteria (LPS: Lipopolysaccharide; LTA: Lipoteichoic acid) (http://www.cehs.siu.edu/fix/medmicro/genmicr.htm)

acids across the OM (Sihavy *et al.*, 2010; Greenwood, 2007; Ryan *et al.*, 2004). The OMPs in *Klebsiella pneumoniae;* OMPK35 and OMP*K*36, act as a channel for antibiotics to pass through these porins to the cytoplasm and losing either has shown to facilitate resistance to cephalosporins (Tsai, *et al.*, 2011).

The periplasm lies between the two membranes (Figure 1.7) and is filled with a fluid called the periplasmic gel and situated between the outer and the inner membranes and considered as the interior part of the cell envelope. The periplasm plays a crucial role as a transporter of sugars and amino acids and because it is densely packed with proteins, it acts to sequester of the harmful RNAse and alkaline phosphatase degradative enzymes. The periplasm is inhabited with periplasm binding proteins and chaperon like molecules, both have different functions. Periplasm binding proteins act as transporter of sugars and amino acids as well as chemotaxis, whereas chaperon like molecules function in envelope biogenesis (Silhavy *et al.*, 2010) such as the movement of synthesised molecules e.g. LPS from the cytoplasm to across the periplasm be assembled on the outer membrane, specific transporters are required; the periplasmic protein LptA, the OM lipoprotein LptE and the β-barrel OM protein LptD. (Ruiz *et al.*, 2009). Chromosomal, plasmid-mediated or inducible β-lactamases present in the periplasm play an important role in protecting the PBPs from β-lactam antibiotics (Sykes & Matthew, 1976).

The cell wall consists of a thin layer of peptidoglycan known as murein 5-10 (nm) linked to the outer membrane via lipoproteins. N-acetylglucosamine and N-acetylmuramic acid molecules represent the main structure of the peptidoglycan layer; moreover, they are cross-linked with penta-peptide side chains (Vollmer *et al.*, 2008). Despite the fact that the peptidoglycan in Gram-negative bacterial cell wall is greatly reduced, it plays a significant role in giving the cell its stability and rigidity and, accordingly, determines cell shape. The reason for this is the composition of glycan chains in the form of N-acetylglucosamine-N-acetylmuramic acid, which is found linked in alternative ways to form murein saculus heteropolymer. The penicillin binding proteins (PBPs) play a major role in the polymerization of the glycan strand that is called transglycosylation. PBPs are the target of β-lactam antibiotics but are protected by β-lactamases in the periplasm (Sauvage *et al.*, 2007).

1.3 Examples of antibiotics used in treatment of infection caused by bacteria

Gram-negative bacteria are a leading cause of life-threatening infections and include nosocomial infections (NI), nosocomial pneumonia (NP), urinary tract infections (UTIs), intra-abdominal infections (IAIs), pediatric bacterial meningitis, septicaemia, neutropenia, community acquired infections (CAIs), and pelvic inflammatory diseases (Lamb *et al.*, 2002; Plosker *et al.*, 1998; Chaudhuri *et al.*, 2011; Baughman, 2009). Since the discovery of antibiotics, many classes of antibiotics have been employed and derivatives of established

antibiotics trialed to overcome increasing resistance. (Table 1.2) (Coates *et al.*, 2002).

Table 1.2 Main classes and examples of antibiotics and β-lactamase inhibitors (according to Coates *et al.*, 2002)

Class	Examples
β-lactams	
Penicillins	Penicillin G, penicillin V, methicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, ampicillin, amoxicillin, carbenicillin, ticarcillin, mezlocillin, piperacillin, azlocillin, temocillin
Cephalosporins	
First generation	Cepalothin, cephapirin, cephradine, cephaloridine, cefazolin
Second generation	Cefamandole, cefuroxime, cephalexin, cefprozil, cefaclor, loracarbef, cefoxitin, cefmetazole
Third generation	Cefotaxime, ceftizoxime, ceftriaxone, cefoperazone, ceftazidime, cefixime, cefpodoxime, ceftibuten, cefdinir
Fourth generation	Cefpirome, cefepime
Carbapenems	Imipenem, meropenem
Monobactams	Aztreonam
β-lactamase inhibitors	Clavulanate, sulbactam, tazobactam
Aminoglycosides	Streptomycin, neomycin, kanamycin, paromycin, gentamicin, tobramycin, amikacin, netilmicin, spectinomycin, sisomicin, dibekacin, isepamicin
Tetracyclines	Tetracycline, chlortetracycline, demeclocycline, minocycline, oxytetracycline, methacycline, doxycycline
Rifamycins	Rifampicin (also called rifampin), rifapentine, rifabutin, bezoxazinorifamycin, rifaximin
Macrolides	Erythromycin, azithromycin, clarithromycin
Lincosamides	Lincomycin, clindamycin
Glycopeptides	Vancomycin, teicoplanin
Streptogramins	Quinupristin, dafloprístin
Sulphonamides	Sulphanilamide, *para*-aminobenzoic acid, sulfadiazine, sulfisoxazole, sulfamethoxazole, sulfathalidine
Oxazolidinones	Linezolid
Quinolones	Nalidixic acid, oxolinic acid, norfloxacin, pefloxacin, enoxacin, ofloxacin/levofloxacin, ciprofloxacin, temafloxacin, lomefloxacin, fleroxacin, grepafloxacin, sparfloxacin, trovafloxacin, clinafloxacin, gatifloxacin, moxifloxacin, sitafloxacin
Others	Metronidazole, polymyxin, trimethoprim

1.3.1 β-lactams

1.3.1.1 Cephalosporins

Cephalosporins are class of antimicrobials used to treat bacterial infections due to Gram-negative and Gram-positive bacteria. Cephalosporins are divided to 1st, 2nd, 3rd, 4th and 5th generations. The 1st generation was first introduced in 1945 as natural product derivatives to disrupt the cell wall by interrupting the synthesis of peptidoglycan causing lysis of bacteria. (Butler & Buss, 2006). Third generation cephalosporins are among the most widely used subclass of antibiotics and include cefotaxime, ceftazidime, and ceftriaxone. This class of antibiotics is administered to treat hospital acquired infections particularly to eradicate infections caused by Enterobacteriaceae e.g. *K. pneumoniae* and *Escherichia coli*.

1.3.1.1.1 Cefotaxime

Cefotaxime has a broad-spectrum of activity and plays an important role in the treatment of Gram-negative bacterial infections in adult and pediatric patients. It is administrated to treat bacterial infections due to skin and soft tissue infections, nosocomial infections, pneumonia, complicated urinary tract infections, meningitis, bone and joint infections and bacteraemia (Adu & Armour, 1995; Plosker *et al.*, 1998; Dajani, 1995).

1.3.1.1.2 Ceftazidime

Ceftazidime is an aminothiazolyl syn-methoxyimino cephalosporin, it is a β-lactam antibiotic has broad-spectrum activity against Gram-negative. Ceftazidime is administered to treat bacterial infections e.g. respiratory tract, genitourinary tract, gynecological, bone and joint, septicaemia, intra-abdominal, bacteraemia, meningitis, skin and tissue and ventilator associated pneumoniae infections (VAPs). (Buijk *et al.*, 2002; Lorente *et al.*, 2007).

1.3.1.1.3 Ceftriaxone

Ceftriaxone was introduced in 1980s and used extensively to treat bacterial infections due to its stability against β-lactamases, particularly produced by members of Enterobacteriaceae. It is used to treat broad range of infections; these include meningitis in adults and infants, acute otitis media, CAIs, uncomplicated gonorrhea, pelvic inflammatory disease, acute pyelonephritis and spontaneous bacterial peritonitis. (Lamb, *et al.*, 2002; Jones, *et al.*, 1998; Diekema, *et al.*, 1999).

1.3.1.2 Carbapenems

Carbapenems are derived from the antibiotic thienamycin which is a natural product of the Gram-positive bacterium *Streptomyces cattleya*. This class of β-lactams includes meropenem, imipenem, ertapenem, and doripenem. Carbapenems are often used as empirical therapy and to treat bacterial infections caused by Gram-negative bacteria that produce resistant

determinants against extended spectrum cephalosporins. Carbapenems are classified into two groups. Group 1 comprises antibiotics that have limited antibacterial activity against non-fermenters Gram-negative bacteria such as ertapenem. Group 2 includes antibiotics active against non-fermenters and recommended to treat nosocomial infections. (Shah & Isaacs, 2003; Livermore and Woodford, 2000; Birnbaum *et al.*, 1985; Ayalew *et al.*,2003; Zhanel *et al.*, 2007; Mohr, 2008).

1.3.1.2.1 Imipenem

Imipenem is *N*-formimodoyl-thienamycin (Figure 1.8) is not used on its own because it is rapidly degraded by dehydropeptidase produced by the human kidney and has an adverse toxic effect on the kidney, therefore imipenem should be co-administrated 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 antibiotic. (Rodloff *et al.*, 2006).

Transpeptidases also known as penicillin binding proteins (PBPs) cross link the peptidoglycan and provide the bacteria with a rigid cell wall are the main targets for imipenem. Imipenem has been shown to inactivate the transpeptidase of PBP-1A, PBP-1B and PBP-2, it moreover, inhibits the D-alanine carboxypeptidase of PBP-4 and PBP-5 in *E. coli*. (Hashizume *et al.*, 1984). Imipenem is a broad-spectrum antibiotic indicated as initial empirical therapy and in treating serious bacterial infections including NI, ventilator

associated pneumonia (VAP), febrile neutropenia (Torres *et al.*, 2000; Zanetti *et al.*, 2003; West *et al.*, 2003; Raad *et al.*, 2003; Cherif *et al.*, 2004), hospital acquired pneumonia (HAP), healthcare associated pneumonia (HCAP), patients hospitalized suffering from intra-abdominal infections, patients with skin and soft tissue infections and lower respiratory tract infections (Neu, 1983; Shah & Isaacs, 2003).

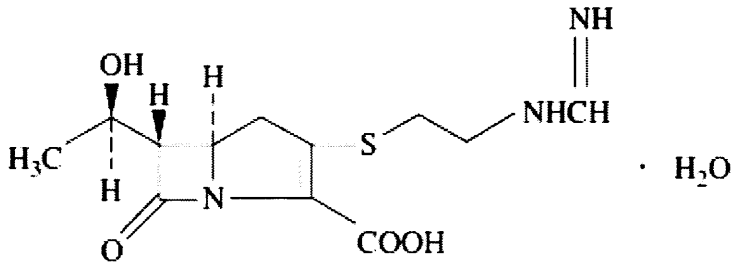

Figure 1.8 (*N*-formimodoyl-thienamycin). (Rodloff *et al.*, 2006).

1.3.1.2.2 Meropenem

Meropenem is a member of carbapenems marketed to eradicate Gram-negative bacterial infections and was approved by the FDA in 1996 (Zhanel *et al.*, 2007; Baldwin *et al.*, 2008). Meropenem binds effectively to penicillin binding protein (PBP) with high affinity, accordingly inhibiting the growth of the micro-organism. It has high affinity to PBPs 2, 3, and 4 of *E. coli* and PBPs 1 and 2 of *Pseudomonas aeruginosa* (Baldwin *et al.*, 2008).

Meropenem is effective in the treatment of several infectious diseases caused by pathogenic bacteria, it is recommended for the treatment of NP, it can also be used as an alternative to other antibiotics such as amikacin (Alvarez Lerma, 2001) or combinations of antibiotics e.g. ceftazidime and tobramycin (Heyland *et al.*, 2008). Meropenem is also very efficacious in treating patients with complicated intra-abdominal infections (CIAI) (Zanetti *et al.*, 1999; Brismar *et al.*, 1995). In one study, 153 patients with septicaemia, meropenem was effective as an empirical therapy, and as effective as ceftazidime with or without amikacin (Baldwin *et al.*, 2008). Meropenem also displays high efficacy in treating adults and paediatric patients suffering from cancer related febrile neutropenia infected with *E. coli*, *Klebsiella* spp and *P. aeruginosa* (Oguz *et al.*, 2006; Kutluk *et al.*, 2004; Feld *et al.*, 2000; Cometta *et al.*, 1996), and patients with bacterial meningitis caused by *K. pneumoniae* and *Haemophilus influenzae* (Odio *et al.*, 1999; Schmutzhard *et al.*, 1995). It is also highly active in treating complicated urinary tract infections (CUTI) (Cox *et al.*, 1995); complicated skin and skin structure infections (CSSSIs) (Fabian *et al.*, 2005) and acute pulmonary infections caused by *P. aeruginosa* in patients with cystic fibrosis (Blumer *et al.*, 2005).

1.3.1.2.3 Ertapenem

Ertapenem has a broad-spectrum activity against Gram-negative bacteria but not non-fermenters as it has limited antibacterial activity and it is recommended for CAIs (Keating & Perry, 2005). Ertapenem is active against

Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases. Ertapenem binds to PBPs, subsequently interferes with bacterial cell wall synthesis and due to occurrence of 1β-methyl substituent, co-administration with cilastatin with ertapenem is not required as ertapenem is stable against renal dehydropeptidase I. (Alhambra *et al.*, 2004)..

1.3.1.2.4 Doripenem

Doripenem was approved by FDA in 2007 to be used to treat CIAI and CUTIs (Paterson & Daryl, DePestel, 2009). Its activity resembles that of meropenem (Jones *et al.*, 2005a; Mushtaq *et al.*, 2004). Doripenem forms a stable acyl-enzymes and causing weakness bacterial cell wall and consequently lead to cell wall rupture as a result of osmotic pressure forces (Stratton, 2005). PBP2 and PBP3 in *P. aeruginosa* and *E. coli* are the prime targets for doripenem (Davies *et al.*, 2008).

Doripenem is very similar to meropenem in the treatment of post-surgical infections (Lucasti *et al.*, 2008); and can be employed to treat CUTIs, pyelonephritis and baseline bacteremia, hospital acquired pneumonia including VAP (Rea-Neto *et al.*, 2008; Chastre, *et al.*, 2008).

1.4 Mechanism of antibiotic action

1.4.1 Introduction

Antibiotics were discovered and introduced as to be used to treat bacterial infections by interrupting the physiological mechanisms inside the bacterial

envelope/cytoplasm that allow normal cellular function. Two main mechanisms of bacterial inhibition are known, bactericidal drugs induce cell death while bacteriostatic drugs act as cell growth inhibitors (Kohanski, *et al.*, 2010). Herein, I will describe the effect of antibiotics on protein synthesis, cell wall and DNA synthesis.

1.4.1.1 Inhibition of protein synthesis

Protein synthesis occurs at the ribosome of bacteria and during phases of synthesis, initiation, elongation and termination, more specifically on the 50S and 30S subunits (Figure 1.9). Inhibitors of protein synthesis differ according to the target site, inhibitors of 50S subunit of Gram-negative bacteria include lincosamide e.g clindamycin and chloramphenicol (Katz & Ashley, 2005). Aminocyclitol family and tetracyclines are among the 30S ribosome inhibitors and include kanamycin, gentamicin and streptomycin. These antibiotics inhibit the bacterial growth by interrupting the access of aminoacyl-tRNAs to the ribosome (Chopra & Roberts, 2001). Protein mistranslation can also occur as a result of the interaction between aminoglycosides and 16S rRNA, such interaction causes alteration in the complex between mRNA and aminoacyl-tRNA at the ribosome and consequently mismatching of tRNA will take place leading to protein mistranslation (Pape *et al.*, 2000).

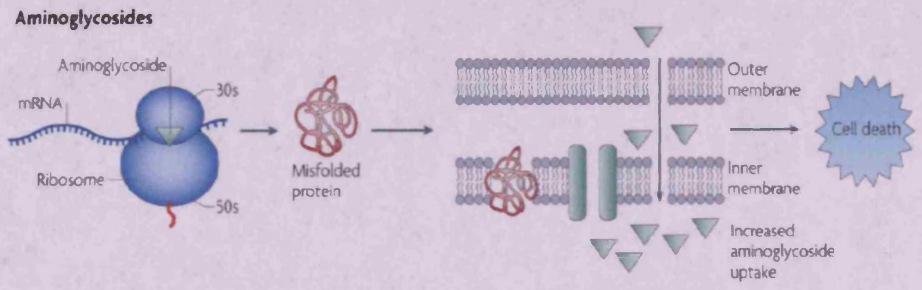

Figure 1.9 Inhibition of protein synthesis by aminoglycosides (according to Kohanski *et al.*, 2010)

1.4.1.2 Cell wall synthesis

The bacterial envelope is enclosed by a covalently cross-linkage of peptidoglycan layers, these layers are composed of peptide β-(1-4)-*N*-acetyl hexosamine. (Bugg & Walsh, 1992). The integrity of the bacterial cell wall is likely to be affected by the degree of peptidoglycan cross-linking (Holtje, 1998). As mentioned previously, β-lactams are the largest group of antibiotics that target the cell wall (Figure 1.10). Glycopeptides also share this target. Carbapenems and cephalosporins are important classes of antibiotics used as a therapy, their mechanism of action is represented in blocking the cross-linking of peptidoglycan units, such blocking is achieved by the inhibition of PBP by means of transpeptidase. (Kohanski *et al.*, 2010).

1.4.1.3 Inhibition of DNA synthesis

Quinolone antibiotics are DNA synthesis inhibitors that act by targeting DNA gyrase that is known as topoisomerase II and topoisomerase IV which is

known as topoIV (Figure 1.11). These antibiotics prevent the rejoining of the DNA strand at the DNA cleavage stage and consequently affect the synthesis of DNA and cause cell death. It has been shown that quinolone antibiotics target topoisomerase II in Gram-negative bacteria e.g. *E. coli* and *Neisseria gonorrhoeae* (Drlica *et al.*, 1978; Kohanski *et al.*, 2010).

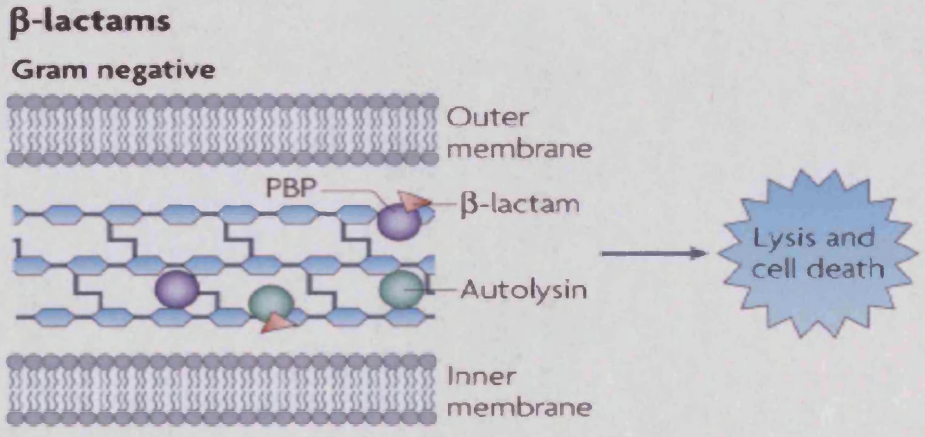

Figure 1.10 Inhibition of cell wall synthesis by β-lactams (according to Kohanski *et al.*, 2010)

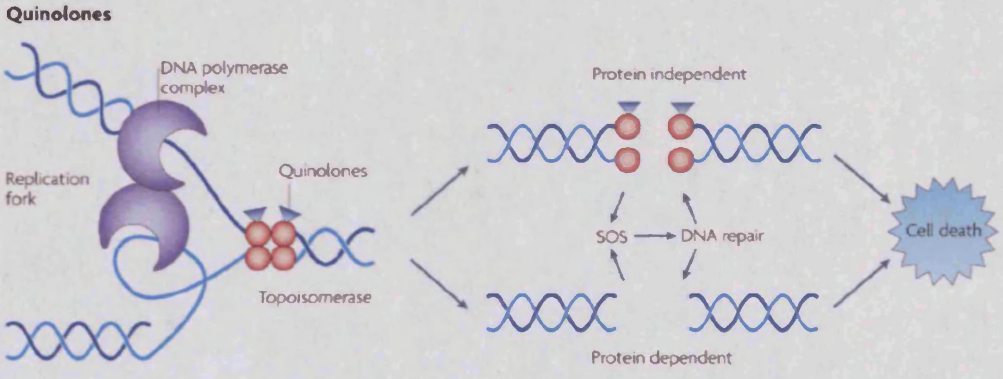

Figure 1.11 Inhibition of DNA synthesis by quinolones (according to Kohanski *et al.*, 2010)

1.5 Mechanism of antibiotic resistance in Gram-negative bacteria

Several factors have been attributed to the ascending level of bacterial resistance to antimicrobial agents used in clinical settings and have led to the emergence of multi-drug resistant strains.

1.5.1 Efflux pump mediated antibiotic resistance

Efflux is considered one major mechanism by which bacteria can expel antimicrobials outside the cell. Efflux pumps are often chromosomally mediated; however, some plasmid mediated pumps have been reported. Five families of efflux pumps were reported, ATP binding cassette superfamily (ABC), the multi-drug and toxic compound extrusion family (MATE), the major facilitator superfamily (MFS), the small multi-drug resistance family (SMR) and the resistance nodulation division superfamily (RND). (Li & Nikaido, 2004; Li & Nikaido, 2009).

Single or multi-drug resistance in *E. coli* is in part attributed to the occurrence of efflux transports in addition to other resistance mechanisms. More than 37 efflux pumps were found in the genome of *E. coli* belonging to different families; seven RND type, seven ABC type, 1 MATE type and 19 MFS. AcrAB is known to work with the outer membrane protein TolC as the combination system shows broad substrate specificity toward β-lactams, chloramphenicol and novobiocin as well as dyes, detergents and organic solvents (Li & Nikaido, 2004). Twelve types of RND type efflux system have

been described as responsible for resistance of *P. aeruginosa* to antimicrobials, detergents, chemicals, molecules, dyes and antiseptics for instance MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OprD and MexXY efflux pumps. MexAB-OprM efflux provides a wide range of resistance to antibiotics, β-lactam, tetracycline, trimethoprim, chloramphenicol with intrinsic resistance toward flouroquinolones (Askoura *et al.*, 2011).

1.5.2 Outer membrane permeability and antibiotic resistance

The outer membrane in Gram-negative bacteria has already been described in (section 1.2). Antibiotics undertake two pathways to penetrate the outer membrane targeting the cytoplasmic membrane; the lipid-mediated pathway and general porin diffusion. Some antibiotics use both ways to enter the cell e.g. tetracycline and quinolones. Hydrophobic antibiotics enter the Gram-negative bacterial outer membrane via the lipid-mediated pathway whereas the hydrophilic antibiotics use porins to reach their target (Delcour, 2009). Gentamicin, kanamycin, erythromycin, rifamycin, fusidic acid and cationic peptides are known as hydrophobic antibiotics able to enter the cell through the outer membrane bilayer (Vaara, 1992; Nikaido, 2003).

Bacteria use the LPS core region as a barrier for hydrophobic antibiotics. Some antibiotics and chemicals play a major role in the sensitivity of bacteria to antimicrobials, e.g. Tris/EDTA and polymyxin B. The target of Polymyxin B is the cytoplasmic membrane; it penetrates the cell and by binding to negatively charged LPS causes destabilisation of the outer membrane, the fatty

acid tail of the antibiotic causes disruption to the membrane integrity leading to the antibacterial action. Resistance of bacteria to polymyxin B is achieved by esterification of the lipid A phosphates by the occurrence of 4 to 6 times of 4-aminoarabinose and more phosphoethanolamine, these compounds lower the negative charge of the LPS leading to more resistance to polymyxin B penetration (Cardoso, 2007; Delcour, 2009).

The term porin refers to β barrel proteins that act as a channel crossing the cell membrane. The classical porins that are known to facilitate the diffusion of molecules are OmpC and OmpC subfamilies; however, some exceptions should be taken into consideration such as PhoE in *E. coli* and OprD of *P. aeruginosa* and others. (http://www.membranetransport.org/). The porin channel provides an entry for β-lactams and fluoroquinolones but Gram-negative bacteria have developed some mechanisms to withstand antibiotics, such as changing porin type or the levels expressed, modification of the target site and synthesis of pore blocking molecules. (Pagés *et al.*, 2008). For example, OmpK35, one of the characteristic porins of *K. pneumoniae* and of the OmpF porin group was replaced with OmpK36 as a result of the exposure to treatment of patients harbouring the *K. pneumoniae* with β-lactam antibiotics (Doménech-Sánchez *et al.*, 2003). In vivo and in vitro evidence show that mutation occurred in OprD of *P. aeruginosa* causing carbapenem resistance in the presence or absence of carbapenemase production (Ochs *et al.*, 2000; Wolter *et al.*, 2004).

1.5.3 β-lactamases

1.5.3.1 Introduction

The term β-lactamase refers to the enzymes produced by micro-organisms that hydrolyses β-lactam molecules and thus singularly or in part enables β-lactam resistance. More than 500 β-lactamase enzymes have been reported to date (www.lahey.org/studies.webt.htm). It is considered the most common β-lactam resistance mechanisms that contribute to wide spread resistance among Gram-negative bacteria (Bush & Jacoby, 2010). β-lactamases differ from one another in substrate profiles which depend on the number and types of antibacterial agents they can inactivate. They also differ in terms of their inhibitor profile. Moreover, the amino acid composition of these enzymes is another factor in distinguishing the similarities and the existence of active hydrolytic parts of the enzyme (Ambler, 1980; Bush, 2010 b). In Gram-negative bacteria, the occurrence of β-lactamase mediated resistance is either expressed chromosomally or is plasmid borne. However, the spread of β-lactamases is frequently associated with plasmid encoded ESBLs, specifically the CTX-M family, and serine carbapenemases KPC and the Metallo-β-lactamases (MBLs) VIM, IMP and NDM-1 (Pitout, 2010). Based on substrate specifications, four major groups of β-lactamases have been identified so far; penicillinases, AmpC-type cephalosporinases, ESBLs and carbapenemases. For the purpose of my thesis, I will primarily focus on ESBLs and carbapenemases rather than the less-extended β-lactamases.

1.5.3.2 Classification of β-lactamases

The importance of the antibiotics penicillins and cephalosporins to treat infectious diseases has led to the focus on exploring the characteristics of enzymes produced by bacteria that hydrolyze these antibiotics. Many bacteria are able to exhibit a new approach to withstand antibiotics, more specifically β-lactams. This is frequently noticed by the insertion of new nucleotide sequences in the genetic context of a particular antibiotic resistance gene or by changing of one or more nucleotides in the nucleotide sequence that lead to different amino acid sequences e.g. TEM group of β-lactamases. Consequently, this may result in a different substrate hydrolysis profile that can lead to a higher level of antibiotic resistance. However, a decrease in antibiotic hydrolysis may also be observed. By 2009 more than 500 unique protein sequences for β –lactamases had been reported (Bush & Jacoby, 2010). β-lactamases have been classified in two ways, the first classification is Ambler classification based on the classification of β-lactamases according to their primary structure (Ambler, 1980), while Bush, Jacoby, Medeiros classification is based on functional characteristics of β-lactamases (Bush *et al.*, 1995).

1.5.3.3 Extended spectrum β-lactamases (ESBLs)

ESBLs are a group of enzymes able to hydrolyze and confer resistance to penicillins cephalosporins, monobactams and oxyimino-cephalosporins that include cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cefepime. These

enzymes do not affect some cephamycins such as cefoxitin and cefotetan. ESBLs have no or little activity towards carbapenems. They are inhibited by the classical β-lactamase inhibitors; clavulanic acid, sulbactam and tazobactam. The majority of ESBLs have been classified under Ambler class A β-lactamases, these enzymes include bla_{SHV} and bla_{TEM} that have evolved from e.g. $bla_{SHV\text{-}1}$ and $bla_{TEM\text{-}1}$ encoding genes. Such derivation is attributed to one or more point mutations occurring on the β-lactamase active site (Paterson & Bonomo, 2005).

ESBLs are often found carried on large plasmids. In addition, a number of antibiotic resistance genes that confer resistance to antibiotics such as aminoglycosides and trimethoprim/sulphamethoxazole are also found on the same plasmids. ESBLs are considered among the largest group of β-lactamase known to activate antibiotics such as penicillins and cephalosporins rendering carbapenems as the last choice for treating infections, this results in more pressure on carbapenems. (Bush, 2010b). CTX-M enzymes are among the wide spread ESBLs, since their first description in 1989 (Bauernfeind *et al.*, 1990), over 120 CTX-M type ESBLs have been discovered to date (http://www.lahey.org/studies/other.asp#table1). CTX-M ESBLs are grouped into five major clusters; CTX-M-1,2,8,9 and 25 (Barlow *et al.*, 2008 & Bonnet, 2004), CTX-M 1 and CTX-M 9 being the most diverse clusters with 31 and 22 variants identified respectively. CTX-M enzymes comprise a wide range of subgroups for instance; CTX-M group1 1, 2 and 9 are known to

include more members of CTX-M variants than CTX-M 8 and 25 e.g, CTX-M-1,3,10,11,12,32,36 and CTX-M-15, CTX-M group 2 encompasses CTX-M-2,20,31,5,6,56,7 and others, CTX-M-9 includes for instance; CTX-M-9,13,14,17,47,48 and CTX-M-55. CTX-M groups 8 and 25 includes only few variants (Novias *et al.*, 2010 & Harada *et al.*, 2008). The dissemination of CTX-M ESBLs is oftentimes associated with the occurrence of Insertion Sequence Common Regions (IS*CR*s) which are found to be located upstream of antibiotic resistance genes and can activate their transmission. The occurrence of CTX-M ESBLs in *E. coli* isolates from nine patients in Norway has been recently assessed. Six of the ESBL genes were $bla_{\text{CTX-M-15}}$ and one $bla_{\text{CTX-M-3}}$. All $bla_{\text{CTX-M-15}}$ bore resemblance to each other in terms of their sensitivity to antimicrobials used with minimum inhibitory concentrations (MICs), ≥ 256 μg/ml and ≥256 μg/ml for cefotaxime and ceftazidime, respectively (Naseer *et al.*, 2007).

1.5.3.4 Carbapenemases

Carbapenems are hydrolysed by carbapenemases produced by Gram-negative bacteria such as members of Enterobacteriaceae and non-fermenters. These enzymes have been classified into three classes according to Ambler classification; class A, B and D. Class A and D are known as serine carbapenemases and Class B are called metallo- β-lactamases (MBLs) (Walsh, 2010).

1.5.3.5 Class A carbapenemases

Class A carbapenemases are also known as group 2f, according to Bush *et al.*, 1995, comprises five phylogenetic groups; NMC, IMI, SME, KPC and GES and are subdivided into chromosomally and plasmid mediated groups. SME, NMC and IMI are chromosomally mediated whereas KPC and GES groups are, in most cases, plasmid mediated. These enzymes possess hydrolytic activity towards most β-lactams including carbapenems, cephalosporins, penicillins, and aztreonam and have been found in Enterobacteriaceae and *P. aeruginosa*. SME-1, SME-2 and SME-3 were chromosomally mediated in *Serratia marcescens* whereas IMI-1, IMI-2 and NMC-A are detected on the chromosome of *Enterobacter cloacae*. GES-2 was found plasmid mediated in *P. aeruginosa*, GES-4 has been detected on a plasmid in *K. pneumoniae* isolated from a Japanese patient whereas GES-5 and GES-6 were plasmid mediated in *E. coli* and *K. pneumoniae* isolated from Greece (Queenan and Bush, 2007; Walsh, 2010).

KPC enzymes are among the plasmid encoded class A serine carbapenemases, mostly from *K. pneumoniae*, and are considered the most frequently detected class A enzymes that have a potent threat to antimicrobials used to treat infections. KPC enzymes were first discovered in *K. pneumoniae* isolated from a patient from North Carolina, USA in 1996. KPC-1 was followed by KPC-2 and, later on KPC3, KPC-4, KPC-5, KPC-6 and KPC-7 as variants of KPC-1 and KPC-2. KPC genes have been reported on plasmid in

Enterobacterial species; *E. coli*, *Salmonella cubana*, *E. cloacae*, *Proteus mirabilis*, and *K. oxytoca*. Self transferable KPC genes have been determined to be transferred to *E. coli*, they have also been detected carried on a 10kb transposon, Tn*4401*, and associated with the insertion sequences IS*Kpn6* and IS*Kpn7* (Nass *et al.*, 2008; Nordmann *et al.*, 2009; Walsh, 2010; Queenan and Bush, 2007).

1.5.3.6 Class D β-lactamases

This class of β-lactamases includes enzymes called oxacillinases, these enzymes hydrolyze cloxacillin, oxacillin, extended spectrum cephalosporins and carbapenems. Oxacillinases such as bla_{OXA-1} and bla_{OXA-10} are among enzymes that show increased hydrolysis of cloxacillin or oxacillin whereas bla_{OXA-11} and bla_{OXA-15} hydrolyse cloxacillin or oxacillin and even oxyimino-β-lactams less efficiently than others. Some β-lactamases can target carbapenems for instance bla_{OXA-23} and bla_{OXA-48} in addition to cloxacillin and oxacillin, these enzymes have been detected plasmid mediated in Enterobacteriaceae (Poirel *et al.*, 2004; Walther-Rasmussen and Hoiby, 2006). Four clusters of oxacillinases are responsible for carbapenem hydrolysis in Gram-negative bacteria; bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58} and bla_{OXA-48} (Walther-Rasmussen & Hoiby, 2006). bla_{OXA-23} cluster comprises two enzymes; bla_{OXA-27} and bla_{OXA-49}. The majority of these enzymes are found in *Acinetobacter* and can be chromosomally or plasmid mediated (Poirel & Nordmann, 2006).

1.5.3.7 Metallo-β-lactamases (MBLs)

MBLs are enzymes capable of readily hydrolysing all β-lactam antibiotics with the sole exception of monobactams. In addition they are not inhibited by the classical serine β-lactamase inhibitors (Walsh *et al.*, 2005), (Jones *et al.*, 2005b; Poirel *et al.*, 2010a; Samuelsen *et* al., 2010; Walsh *et al.*, 2005). At molecular level, MBLs are a disparate group of proteins, they are classified to three classes; B1, B2 and B3 based on sequence identity and other structural features. Classes B1 and B3 possess two zinc ions in their active sites and class B2 possesses only one zinc ion. The widely spread enzymes belong to class B1, these enzymes posses the key zinc coordinating residues of three Histidine and one cysteine such as; IMP, VIM, GIM and SPM-1, class B2 include enzymes that posse asparagine instead of Histidine (Walsh *et al.*, 2005) Most MBL genes are located on mobile genetic elements, the majority of these MBL encoding genes are carried in the form of gene cassettes on class 1 integrons and/or Tn*402*-type transposons (Marchiaro *et al.*, 2010; Poirel *et* al., 2010b; Borgianni *et al.,* 2011; Lee *et* al., 2005; Castanheira *et* al., 2004; Santos *et al.*, 2010) whereas some of these genes are associated with insertion sequences such as IS*CR4* (bla_{SPM-1}), (Salabi *et al.*, 2010; Poirel *et al.*, 2004) and IS*26*/Tn*3* transposon (Yong *et al.*, 2009) which can facilitate their global spread. MBLs have been reported worldwide in non-fermenting Gram-negative bacteria (Osano *et al.*, 1994) and more recently in Enterobacteriaceae (figure 1.12) (Kumarasamy *et al.*, 2010)

The continuous emergence of MBLs and their association with MDR phenotypes in Gram-negative bacteria are considered major threats in the treatment of infectious diseases. To date 9 acquired MBLs have emerged worldwide (Figure 1.12); IMP (Osano *et al.*, 1994), VIM (Lauretti *et al.*, 1999), SPM-1 (Toleman *et al.*, 2002, GIM-1 (Castanheira *et al.*, 2004), SIM-1 (Lee *et al.*, 2005), AIM-1 (Gupta, 2008), KHM-1 (Sekiguchi *et al.*, 2008), NDM-1 (Yong *et al.*, 2009) and DIM-1 (Poirel *et al.*, 2010) genes in addition to the novel TMB-1 that was recently detected in Libya (see chapter 6).

The prevalence of carbapenem resistance strains of *P. aeruginosa* has been reported in China during the period of 2004 to 2005. *P. aeruginosa* strains have been collected from different cities in China including a large teaching hospital in Beijing and data shows that 10 % of all imipenem resistant *P. aeruginosa* carry bla_{VIM} type MBLs. 12 out of 14 strains of *P. aeruginosa* were positive for class 1 integrons carrying bla_{VIM-2}. These results reveal that bla_{VIM-2} type MBL genes disseminated horizontally in China between different cities, due to patients transfer among cities inside the country. (Yu *et al.*, 2006).

Numerous strains of Gram-negative bacteria possess chromosomes that have become a mosaic as a result of the horizontal gene transfer and the vertical inheritance of genes (Waldor, 2010). Four mechanisms by which antibiotic resistance genes can horizontally be mobilised from a chromosome to a

plasmid are integrons, transposons, Integration Conjugative Elements (ICE) (Partridge, 2011) and Insertion Elements (Toleman & Walsh, 2011).

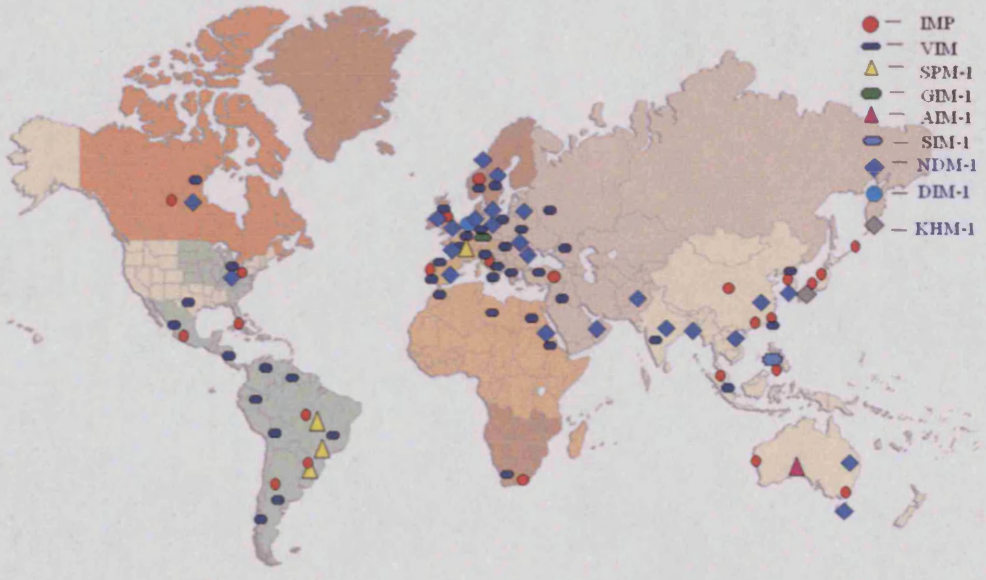

Figure 1.12 Global emergence of MBLs (modified from Walsh, 2010) .

1.6 Global emergence of clinical antibiotic resistant Gram-negative bacteria

Gram-negative bacteria, more importantly those belong to Enterobacteriaceae and non-fermenters such as *P. aeruginosa* and *Acinetobacter baumannii*, are among the most causative agents of hospital and community acquired infections. Extended-spectrum cephalosporins, fluoroquinolones and carbapenems are among the main therapeutic choices. The continuous pressure of β-lactam antibiotics in hospitals has exacerbated the selection for consecutive generations of β-lactamases – ESBLs followed by carbapenemases (Chouchani *et al.*, 2011; Coque *et al.*, 2008). The problem

becomes worse when such resistance occurs by horizontal gene transfer or mediated by conjugative plasmids as it is the case generally for ESBLs causing resistance to extended-spectrum cephalosporins. For example, CTX-M-type ESBLs have been detected in Europe with $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CTX\text{-}M\text{-}9}$ carried on a variety of different Inc-type plasmids and sizes (Figure 1.13) (Livermore *et al.*, 2007).

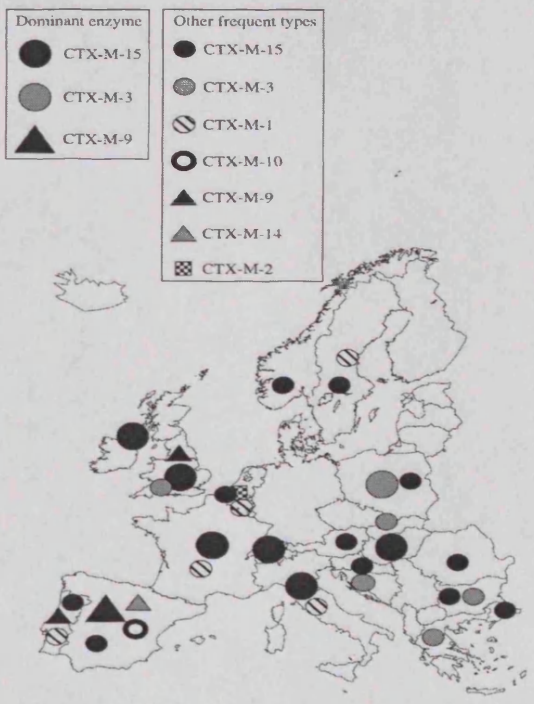

Figure 1.13 The emergence of CTX-M type ESBLs in Europe. (Livermore *et al.*, 2007)

According to the antimicrobial resistance surveillance conducted in Europe between 2006-2009, the recent global emergence of antimicrobial resistance of *K. pneumoniae*, *E. coli* and *P. aeruginosa* in Europe showed that there is an

increasing trend in the resistance of these micro-organisms to antimicrobials used. The surveillance showed that *E. coli* isolates collected from European countries exhibited high resistance to aminopencillin, extended-spectrum cephalosporins and aminoglycosides. European antimicrobial resistance surveillance (EARS-Net) data also shows a continuing increase in flouroquinolone resistance. High proportion (85-100%) of *E. coli* isolates resistant to extended-spectrum cephalosporins were due to ESBLs indicating the high prevalence of ESBL producing *E. coli* in European hospitals (http://ecdc.europa.eu/en/publications/Publications/1011_SUR_annual_EARS_Net_2009.pdf).

A high proportion of resistance of *K. pneumoniae* to extended-spectrum cephalosporins, fluoroquinolones and aminoglycosides is evident. *K. pneumoniae* isolates from two countries; Greece and Cyprus in the Mediterranean Gulf also show high resistance to carbapenems. Half of the countries involved in the surveillance program reported the incidence of multi-drug resistant (MDR) *K. pneumoniae* to extended-spectrum cephalosporins (Figure 1.14), aminoglycosides and fluoroquinolones whereas northern European countries such as Denmark and Norway reported an increasing trend of resistance to specific classes of antibiotics whilst emergence of resistance in UK showed a consistent reduction (http://ecdc.europa.eu/en/publications /Publications /1011_SUR_ annual_ EARS_Net_2009.pdf).

With respect to EARS-Net data on *P. aeruginosa* in Europe, data on the resistance trends from the eastern and southern parts of Europe show a higher proportion of antibiotic resistance. Overall, of 8129 *P. aeruginosa* isolates collected from the 28 countries participating in the surveillance, 1541 have shown resistance to carbapenems; imipenem and meropenem (Figure 1.15). (http://ecdc.europa.eu/en/publications /Publications /1011_SUR_ annual_ EARS_Net_2009.pdf).

Carbapenems were introduced as a first line therapy to treat infections caused by non-fermenters in the 1980s, they have also been used for ESBL-producing Enterobacteriaceae after the increasing trend of resistant enterobacterial species to 3rd generation cephalosporins. Since then acquired carbapenemases started to appear and attracted increasing attention most notably MBLs and to lesser extent other carbapenemases such as class A. Since the discovery of MBLs, 9 of these enzymes with their variants have been reported in Latin America, USA, Europe, Africa, Southern Asia, India and Australia and recently TMB-1 in Libya (see chapter 6). Recently MBLs were found in *K. pneumoniae*, *E. coli* and *E. cloacae* such as bla_{NDM-1} that first emerged in India, followed by the UK, and currently has been detected in many countries worldwide (Pfeifer *et al.*, 2011; Chen *et al.*, 2011; Wu *et al.*, 2010; Perry *et al.*, 2011; Solé *et al.*, 2011; Jovcic *et al.*, 2011; Yamamoto *et al.*, 2011).

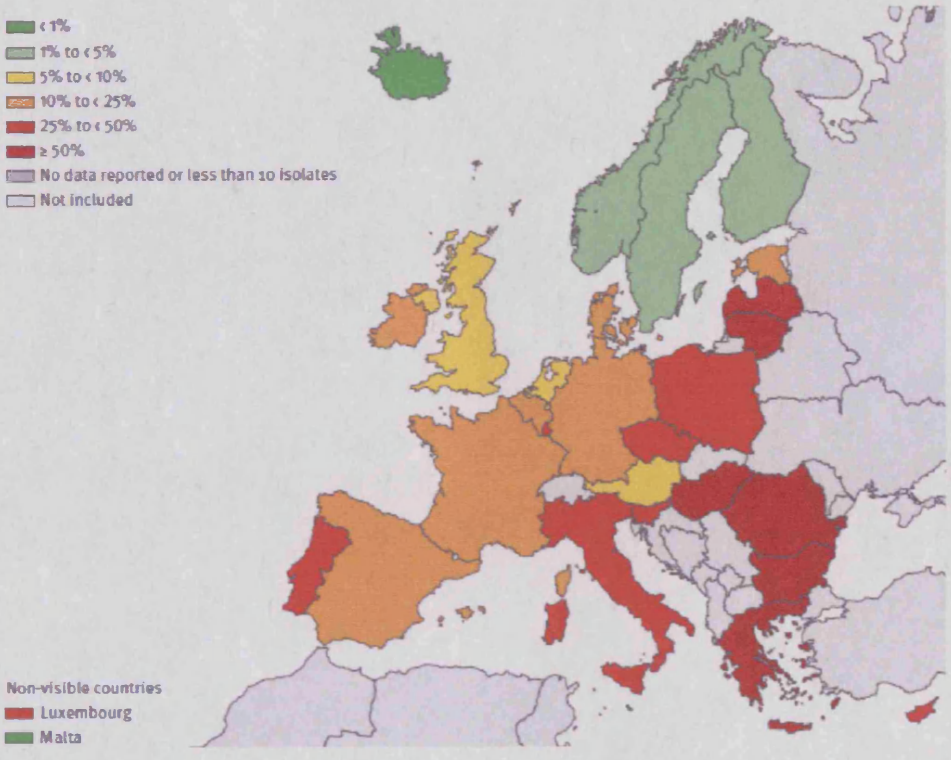

Figure 1.14 The occurrence of *K. pneumoniae* resistant to 3rd generation cephalosporins in Europe (http://ecdc.europa.eu/en/publications/Publications/1011_SUR_ annual_ EARS_Net_2009.pdf**)**

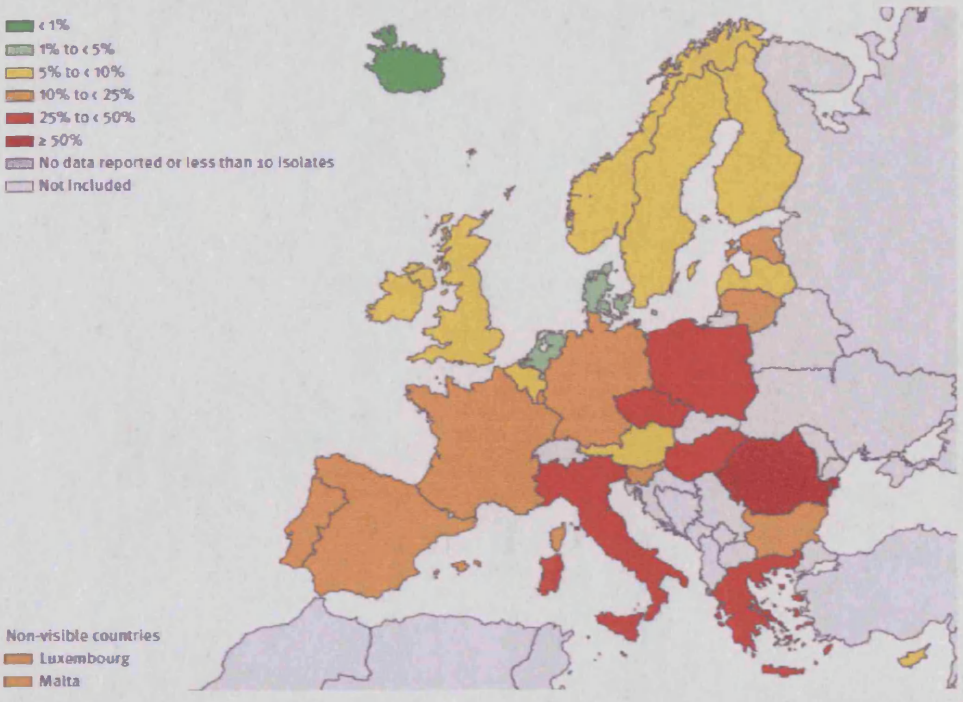

Figure 1.15 The occurrence of *P. aeruginosa* resistant to carbapenems in Europe(http://ecdc.europa.eu/en/publications /Publications /1011_SUR_ annual_ EARS_Net_2009.pdf**)**

1.6.1 Evolution of antibiotic resistance in Gram-negative bacteria

Antimicrobial resistance surveillance programs are vital and considered a longitudinal means of detecting changes in resistance to antimicrobials in clinically important pathogenic bacteria. These programs include the Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) (Turner et al., 1999), SENTRY (http://www.jmilabs.com/surveillance/), Intensive Care Antimicrobial Resistance Epidemiology (ICARE) (Perasso *et al.*, 1999), European Antimicrobial Resistance Surveillance (EARSS)

(http://www.hps.scot.nhs.uk/haiic/amr/earsurveillance.aspx) and others. The monitoring of these programs provides information on the increasing or decreasing level of antibiotic resistance rate worldwide. It moreover offers a guide for empirical treatment regimens. The massive use of antimicrobial agents is the leading cause of the prevalence of antibiotic resistant strains in community and hospital settings.

As an example, a longitudinal study was carried out from 1993 to 2004 aimed to assess the resistance rates of Gram-negative bacilli that cause infections in the intensive care units in the United States (Lockhart *et al.*, 2007). Forty three US states in addition to Columbia were included in this study and 74,394 isolates belong to 11 species of Gram-negative bacteria were collected and tested against 17 antibiotics. The results showed that 22.2 % of all Gram-negative isolates were *P. aeruginosa* followed by 18.8 % *E. coli* and 14.2 % *K. pneumonia*, with additional low percentages of other Gram-negative bacteria. Furthermore, *P. aeruginosa* was the highest among UTIs with 29.9 %. *E. coli* represented the highest among urine isolates with 42.4 %, while it counted as 23.9 % in the blood. Antibiotic susceptibility testing revealed that the highest resistance rate have been recorded for ampicillin-sulbactam, with five-fold increase in the resistance of *P. aeruginosa*, while evaluation of the rate of multi-drug resistance between 1993 and 2004 showed that a longitudinal increase in MDR has been observed (Table 1.3). It has been noticed that there is an association between fluoroquinolone usages as a

therapy and resistance, because the prolonged use of these antibiotics have attributed to the rise of ESBL producing *E. coli* and *P. aeruginosa.* (Lockhart *et al.*, 2007). According to the CDC, MDR is defined as the resistance of bacteria to ≥ 3 classes of antibiotics.

Table 1.3 Longitudinal increase in multi-drug resistance in USA (Lockhart *et al.*, 2007)

Organism	1993		2004	
	No. of MDR isolates/total no. of isolates	% of MDR isolates	No. of MDR isolates/total no. of isolates	% of MDR isolates
P. aeruginosa	13/769	1.7	93/1004	9.3
E. coli	0/724	0	16/808	2
K. pneumoniae	26/513	5.1	84/633	13.3
E. cloacae	13/397	3.3	24/406	5.9
Acinetobacter spp.	19/285	6.7	101/338	29.9
E. aerogenes	6/213	2.8	0/154	0
P. mirabilis	1/174	0.6	1/142	0.7
C. freundii	5/95	5.3	7/63	11.1

Another study conducted in Sierallana Hospital in Spain sought factors that may have an additional effect on patients admitted with bacteraemia. Blood samples from 15045 patients were collected to determine the causative agents of bacteraemia in the period from 1997 to 2005. Antibiotic susceptibility tests were performed using the following antimicrobials; ampicilin, amoxicillin/clavulanate, pipracillin/tazobactam, cefotaxime and trimethoprim/ sulfamethoxazole. 14.9 % of the patients had positive blood cultures, of which, 4.4 % of isolates were *E .coli*. It has been reported that the factors that attributed to the occurrence of bacteraemia in this hospital were; MDR *E.coli*, ESBL producing *E. coli*, age of patients, time of treating with antibiotics and the presence of severe sepsis, which collectively had a role in the morbidity due to *E. coli* infections (Peralta *et al.*, 2007).

The activity of meropenem and 11 other antimicrobial agents including third generation cephalosporins has been assessed in the USA for 10 years in the period between 1999-2008 to demonstrate any increase or decrease in the rate of antibiotic resistance. A steady increase in the resistance rate of ciprofloxacin was observed among *E. coli* (Figure 1.16), an increase in the resistance of *K. pneumoniae* strains was detected for meropenem, ceftazidime, piperacillin/tazobactam, tobramycin and ciprofloxacillin from 2004 to 2007, however the resistance rate to these drugs slightly decreased in 2008 (Figure 1.17). (Rhomberg and Jones, 2009)

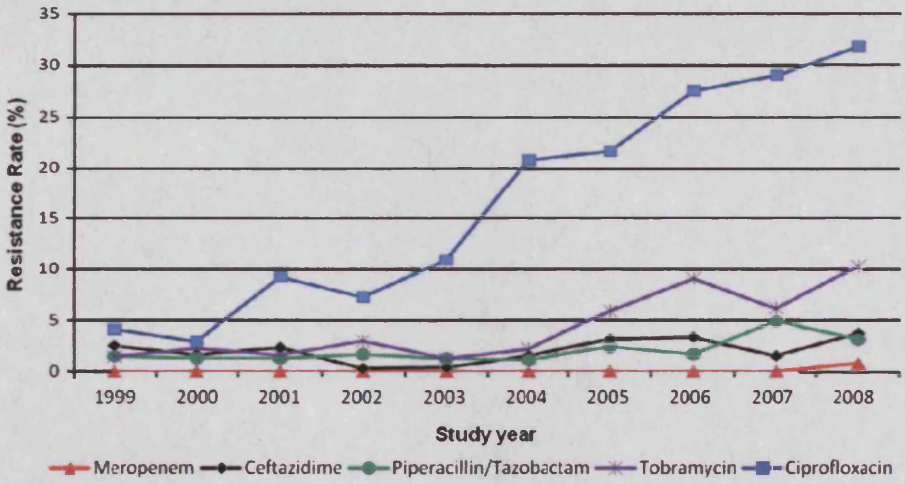

Figure. 1.16 Annual rate of antimicrobial resistance among *E. coli* isolates (4394 strains) tested against selected agents from the MYSTIC Program (1999–2008). (According to (Rhomberg and Jones, 2009)

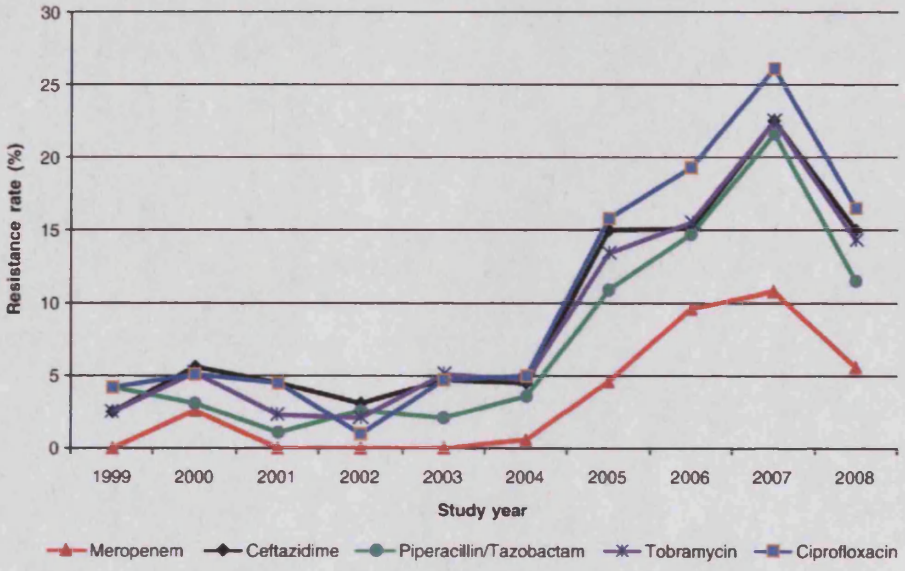

Figure. 1.17 Annual rate of antimicrobial resistance among *K. pneumoniae* isolates (2694 strains) tested against selected agents from the MYSTIC Program (1999–2008). (According to Rhomberg and Jones, 2009)

In Arabia, data on antimicrobial resistance is lacking. However, in Tunisia the appearance of $bla_{CTX\text{-}M}$ family occurred in 2005 after the identification of $bla_{CTX\text{-}M\text{-}27}$ associated with IS*Ecp1* in *Salmonella enterica* and continued to appear in 2006, 2009 and 2010. MBLs were only found in three isolates, $bla_{VIM\text{-}2}$ in two isolates of *P. aeruginosa* and $bla_{VIM\text{-}4}$ produced by *K. pneumoniae* in addition to other ESBL genes. Oxacillinases started to be reported in 2007 when $bla_{OXA\text{-}18}$ was detected in *P. aeruginosa* and different OXA enzymes continued to emerge up to the discovery of $bla_{OXA\text{-}48}$ the carbapenem hydrolysing enzyme in *K. pneumoniae* in 2010. (Figure 1.18) (Chouchani *et al.,* 2011). The first report of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}3}$ in *E. coli*, *K. pneumoniae* and *E. cloacae* isolated from two hospitals in Bejaja, Algeria appeared in 2006 (Touati *et al.*, 2006) followed by detection of $bla_{CTX\text{-}M\text{-}15}$ in *K. pneumoniae* and *E. coli* from hospital environment (Touati *et al.*, 2007) and in *Salmonella enterica* isolated from patients in Algeria (Touati *et al.*, 2008). $bla_{VIM\text{-}19}$ was reported as a novel MBL found in Enterobacteriaceae in Algeria (Robin *et al.*, 2010). Mechanism of antibiotic resistance in clinical isolates of *P. aeruginosa* from patients admitted to the University affiliated hospital of Tlemcen in Algeria was due to the production of $bla_{OXA\text{-}10}$ and bla_{TEM110} (Drissi *et al.*, 2008). The first description of CTX-M producing Gram-negative bacteria in Egypt was from clinical isolates of *E. coli* in 2006 (Mohamed Al-Agamy *et al.*, 2006) while the first report of bla_{TEM} and bla_{SHV} appeared in 2009 (Ahmed *et al.*, 2009), showing the lack of research on this subject. $bla_{NDM\text{-}2}$ was the only MBL detected in *A. baumannii* from Egypt

(Kaase *et al.*, 2011). Furthermore, work on bla_{OXA} enzymes from Egypt appeared only in 2011 (Ahmed and Shimamoto, 2011). Plasmid mediated bla_{TEM-3} has been detected in *S. typhimurium* isolated from patients admitted to the IbnRochd University hospital of Casablanca (AitMhand *et al.*, 2002), moreover, bla_{TEM} and bla_{SHV} were reported from *E. coli* and *K. pneumoniae* isolated from community acquired urinary tract infections from three Moroccan cities; Casablanca, El Jadida and Settat (Barguigua *et al.*, 2011). Research on ESBLs, oxacillinases and MBLs started to appear in 2011 showing the lack of focus on antibiotic resistance in Gram-negative bacteria (Barguigua *et al.*, 2011; Porton *et al.*, 2011 & Poirel *et al.*, 2011).

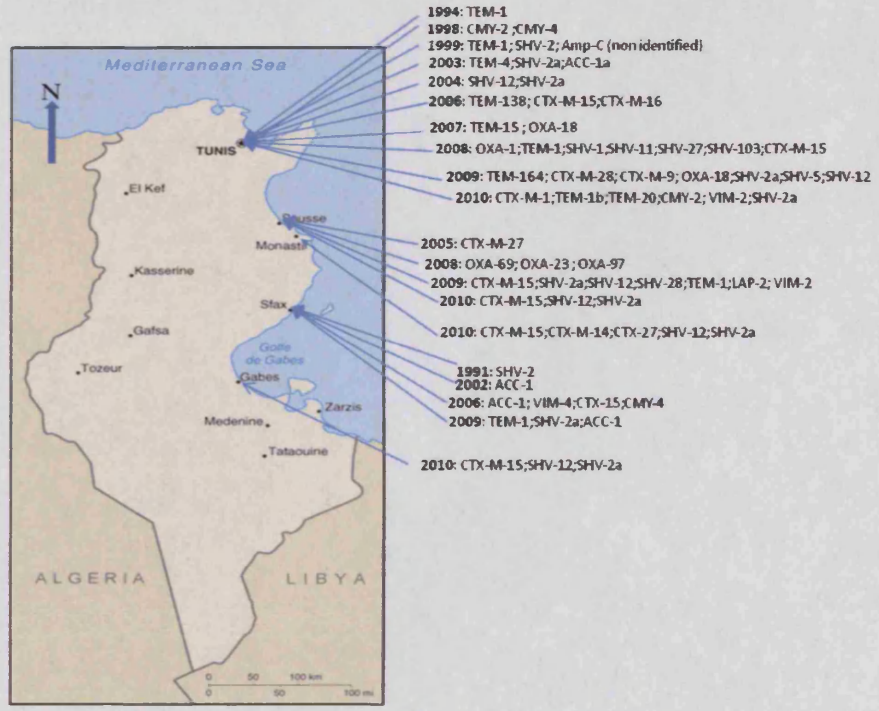

Figure 1.18 The occurence of β-lactamases, ESBLs and carbapenemases in Tunisia (Chouchani *et al.*, 2011)

1.7 DNA structures that spread antibiotic resistance

1.7.1 Plasmids in multi-resistant Gram-negative bacteria

Plasmids are extra-chromosomal DNA found in the cytoplasm of bacteria as independent genetic moieties capable of autonomously reproducing copies of the same plasmid within the cell in the presence of mechanisms to control plasmid copy number and the stability of plasmid inheritance. Plasmids carry essential genes for establishing and directing replication. Furthermore, they do not normally have any functional contribution that is necessary for the cell or cell growth. Plasmids are circular and sometimes linear double stranded DNA segments that normally replicate without affecting the circular chromosome (Carattoli *et al.*, 2005). Plasmids are known to carry genes code for detoxification, ecological interactions, virulence and antibiotic resistance. Plasmids can confer and mobilize resistance to antimicrobials by acquiring resistance genes via horizontal gene transfer and consequently increase the genetic diversity of bacteria.

Resistance genes in Enterobacteriaceae have different constraints for host ranges depending on the plasmids that carry them. It is supposed that genes carried on IncP, IncA/C and IncQ can move to genera of Enterobacteriaceae in addition to *Pseudomonas* and even Gram-positive bacteria due to their larger host range. Other plasmids such as IncFII have a limited host range restraining the transferability of antibiotic resistance genes located on these plasmids, for instance $bla_{\text{CTX-M-15}}$ does not have the ability to move to non-fermenters such

as *Acinetobacter* and *Pseudomonas* and only limited for Enterobacteriaceae (Carattoli, 2009; Smillie *et al.*, 2010).

1.7.2 Pathogenicity islands (Multi resistance in bacteria)

The multi-resistance genotype can reflect the occurrence of resistance islands that include a considerable number of resistance markers and are known as genomic islands (Schmidt & Hensel, 2004). Several bacterial species have shown that the multi-drug resistance phenotypes were mainly attributed to the incidence of resistance islands. These isolates include; *Shigella flexneri*, *S. enerica*, *Vibrio cholera* and *Staphylococcus aureus* with genomic islands sized 20 to 60 kb (Dobrindt *et al.*, 2004).

One of the first resistance islands to be fully characterised from genomic sequencing was that by Fournier *et al.* that reported an 86kb island from *A. baumannii*. This strain, AYE, included 45 antibiotic resistance genes, 25 of which belong to β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, trimethoprim, chloramphenicol, rifampicin and sulphonamides. Several antibiotic resistance genes were previously reported in *Acinetobacter* spp for instance $bla_{\text{OXA-10}}$ and $bla_{\text{VEB-1}}$, *aac*3, *aad*A1/B and *dhfr*1, were also found in this island whereas some other resistance genes had not been reported in *Acinetobacter* species before such as *aac*6, *tet*A, *cml*A, *dfr*X and $bla_{\text{OXA-69}}$. *aac*6 confers resistance to aminoglycosides except gentamicin, *tetA* is a tetracycline resistance gene, *cml*A encodes the multidrug efflux pump Cmr/MdfA, *dfr*X confers resistance to trimethoprim and $bla_{\text{OXA-69}}$ is a class D

β-lactamase found to weakly hydrolyse imipenem and meropenem (Figure 1.19). The island also showed the incidence of three class 1 integrons with 14 gene cassettes embedded within these integrons (Fournier *et al.*, 2006).

Transposons and insertion sequences were detected in the island and showed the occurrence of 22 ORFs encoding transposases, 4 transposons, 2 truncated transposons Tn*5393* and Tn*1721* and two Tn-like transposable elements. The occurrence of this massive number of antibiotic resistance markers, antiseptics and mercury resistance genes in one strain shows how complex the genetic pool can be. (Fournier *et al.*, 2006).

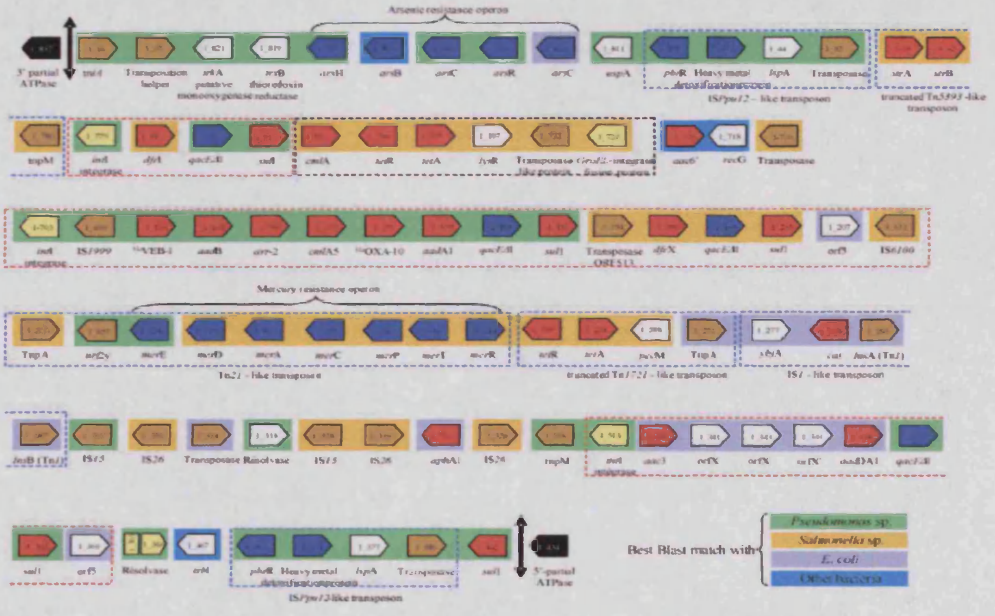

Figure 1.19 Genomic island of *A. baumannii* AYE according to (Fournier, PE. *et al.*, 2006)

1.7.3 Transposons

Some transposons contribute to the movement of antibiotic resistance genes as part of class 1 integrons. These transposons include the Tn*3* family, the Tn*5053* family and Tn*402*-like transposons. These families differ from each other in terms of structure and transposase genes carried by these transposons. The Tn*3* family is composed of two subgroups; Tn*3*-like and Tn*21*-like transposons, they share the same 38bp Inverted Repeat IR, transposase gene (*tnpA*), a resolution site (*res*) and resolvase gene (*tnpR*). These transposons carry antibiotic resistance genes as part of class 1 integron, moreover, they carry mercury resistance genes and genes specific for transposition functions. Unlike the Tn*3* family of transposons, the Tn*5053* family and Tn*402*-like transposons are responsible for carrying and spreading antibiotic resistance genes captured by class 1 integrons. Two major steps have been proposed to elucidate the mechanism by which class 1 integron has become part of the Tn*402*-like transposons. The first step was by inserting the integron inside the Tn*402*-like transposons while the other step suggest the formation of the conserved segment (*qacEΔ/sulI*) followed by the loss of part of *tni*. (Toleman *et al.*, 2007; Sajjad *et* al., 2011). Tn*402*-like differ from the Tn3 family in having three transposase genes; *tniA*, *tniB*, *tniQ*, the resolution site *res* is located between these genes and the resolvase gene *tniR* and sometimes called *tni*C gene. Tn*402*-like transposons are increasingly reported carrying antibiotic resistance genes in the form of gene cassettes embedded in class 1 integrons. (Partridge, 2011).

Transposons such as Tn*5090*/Tn*402* carrying bla_{VIM-2} was detected in a clinical isolate of Indian *P. aeruginosa*. Sequencing of the full transposon, including the integron showed that this structure is very much like the American and Russian bla_{VIM-2} integron structures harbouring *aacA7*, bla_{VIM-2}, *dhfrB5* and *tni*C. All three integrons had the same variable region structure and lacked the conserved segment considered a character of class 1 integrons harboured by TN*5090*/Tn*402* transposons, resulting from excision and acquiring of gene cassettes. (Toleman *et al.*, 2007).

1.7.4 Integrons:

Integrons are genetic elements found in most cases, plasmid mediated and recently some large integrons were detected on the chromosome. Integrons carried on plasmids are responsible for the incorporation of antibiotic resistance genes known as gene cassettes inside the integrons and as a result of this integration they enhance the expression of the gene conferring resistance to antimicrobials. (Walsh, 2006; Mazel, 2006). The first integron was detected in Gram-negative bacteria as a mechanism by which integrons in cooperation with transposons can express multi-resistance phenotype. Integrons are classified in two kinds; mobile integrons and superintegrons. Mobile integrons are always plasmid located and are divided into five classes; class 1 integrons originated from Tn*402* and is found inserted in Tn*21* (mazel, 2006). Class 1 integrons are largely associated with acquiring and mobilising antibiotic resistance genes and are counted as the main responsible system for such

occasion in Enterobacteriaceae. The wild-type class 1 Integron is composed of two sequences; 5 conserved sequence which is also known as (5′CS) and 3 conserved sequence (3′CS) where 5′CS represents the Intgrase gene and 3′CS comprises quaternary ammonium compound resistance gene (qacΔE1) and sulphonamide resistance gene (*sul*1), respectively. (Cambray *et al.*, 2010).

This class of mobile integrons is responsible for conferring resistance to some β-lactams such as aminoglycosides, trimethoprim, rifamycin, erythromycin, streptothricin, chloramphenicol, fosofomycin, quinolones and antiseptics. The integrase (*Int*I) gene is the functional constituent of the integron; it encodes an enzyme called site-specific tyrosine recombinase and it operates to excise and integrate gene cassettes on the attachment site (*attC*). It is called recombination process (Walsh, 2006). The majority of gene cassettes are promoter-less and requires Pc promoter embedded on the integrase gene or attI site. (Cambray *et al.*, 2010).

Class 2 Integrons are found embedded on large transposon called Tn*7* and despite the fact that class 2 integrons encode for non-functional proteins due to a nonsense mutation in codon 179, they are likely to confer resistance to six antibiotics, whereas Class 3 integrons are less frequent than class 2 integrons. Class 4 and 5 are mainly related to trimethoprim resistance in *V. cholerae*. Super Integrons are larger than mobile Integrons and they have been described in *V. cholerae* and because of their location on the chromosome; alone, they

are not mobile and consequently are not capable of mobilising genes (Mazel, 2006).

Class 1 integrons in particular play an important role in disseminating carbapenemase encoding genes such as MBLs in addition to other antibiotic resistance determinants in Enterobacteriaceae and non-fermenters. The most virulent and crucial factors for high levels of resistance to carbapenems particularly MBLs were identified as carried on class 1 integrons, for instance; bla_{NDM-1}, bla_{VIM-1}, bla_{VIM-2}, bla_{IMP} and bla_{DIM-1} (Poirel *et al.*, 2010; Yong *et al.*, 2009; Walsh *et al.*, 2005; Zhao *et al.*, 2009). Class 1 integron was also found to have contributed to dissemination of antibiotic resistance genes to unrelated clinical isolates in Brazil where it has been detected carrying bla_{IMP-1} and a new aminoglycoside resistance gene, *aac(6')-31* in *P. putida*, different isolates of *A. baumannii* and *Acinetobacter* sp.(Mendes *et al.*, 2007)

Integrons have also been detected in bacterial strains collected from manured soil with increased prevalence of integrons after slurry application. Class 1 and 2 integrons were determined in *Acinetobacter*, *Aerococcus*, *Bacillus*, *Enterococcus*, *Pseudomonas* and Enterobacteriaceae (Byrne-Bailey *et al.*, 2011). Class 1 integrons were also identified in sewage treatment plants occurring at different levels in affluent water, activated sludge and effluent water. It has been shown that 57 isolates out of 189 isolates belonging to *E. coli*, *Klebsiella*, *Aeromonas salmonicida*, *A. veronii* and *A. media*, were identified carrying class 1integrons (Ma *et al.*, 2011).

1.7.5 Insertion Sequence Common Regions (ISCRs)

Common Regions (CRs) have been discovered since the mid-1990s as being associated antibiotic resistance genes. It has a size of 2154 bp and incorporated an open reading frame, *orf513*, that was found inserted adjacent to class 1 integron and beside the *sul1* gene (Toleman *et al.*, 2006a). They comprise orf*513* and 33 bp sequence of DNA and they argued that it might play a role in what is called recombination crossover site (RCS). Common regions can promote the expression of some resistance genes in *E. coli*, *K. pneumoniae* and *A. baumannii* and these genes are: *qnrA*, $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-2}}$ and *dfr*A10 (Rodriguez-Martinez *et al.*, 2006).

Common Regions or Insertion Sequence Common Region (IS*CR*) have now become an established mechanism of gene movement. It is proposed that *ISCR* possess two ends, *ori*IS and *ter*IS as insertion and termination sites of *ISCR*, respectively. These insertion sequences have been found as truncated parts at the right side of 3′CS of class 1 Integron and associated with two genes; *qac* and *sul*. Furthermore, this insertion sequence has been found without *ter*IS – providing evidence that a deletion event occurred. Misreading of *ter*IS and passing through many events of transcriptions and translocation resulted in the development of these "complex class 1 Integron", together with misreading and homologous recombination has resulted in genes *qac* and *sul* added to the end of 3′CS (Toleman *et al.*, 2006a).

Many derivatives of IS*CR*s have been discovered and associated with the mobilisation of antibiotic resistance genes. These insertion elements were firstly described in In*6* and In*7* class 1 Integrons. *ISCR1* can carry trimethoprim resistance genes such as *dfrA*23 and *dfr*A18, also they have been found to be associated with quinolones resistance (Stokes *et al.*, 1989). *ISCR1* has been detected upstream of *qnr*A in class 1 Integrons and virtually all isolated genes were identical in spite of their country of origin. IS*CR*1 plays a major role in the resistance of Gram-negative bacteria to aminoglycosides where it has been detected upstream of *arm*A genes. Additionally, IS*CR*1 is associated with ESBLs that inhibit the activity of the antibiotic cefotaxime and class A β-lactamases such as $bla_{\text{VEB-3}}$, $bla_{\text{PER-3}}$, $bla_{\text{CMY-1}}$ and $bla_{\text{CMY-9}}$. IS*CR*2 is also widely disseminated and associated with resistance islands such as SXT via orfA. Despite the fact that IS*CR2* is not associated with class 1 integrons, it has been found associated with *sul*2 gene. IS*CR3* seems to be more specific for *Salmonella* genomic island 1 genetic element (SGI 1 element) and erythromycin gene (*erm*). However, it has also been proposed that it is associated with the resistance of *Stenotrophomonas maltophilia* to trimethoprim/sulfamethoxazole. (Toleman *et al.*, 2006b)

1.7.6 Insertion Sequences

Insertion sequences are considered as the simplest bacterial mobile DNA interms of their structure, they comprise more than 19 families, they have different sizes but in general they range between 600 to 3000 bp. They consist

of one or more open reading frame that code for transposase proteins flanked with short sequences of inverted repeates (Wagner *et* al., 2007) IS*Ecp1*B is another paradigm of insertion sequences that is associated with mobilisation and expression of some antibiotic resistance genes. It is characterised by several features; it can express and mobilise as well as disseminate the cefotaxime resistance gene $bla_{\text{CTX-M-19}}$. Promoter sequences, which are located close to its inverted right repeat (IRR), can also facilitate expression of genes. IS*Ecp1B* has been found associated with $bla_{\text{CTX-M-19}}$ in a strain of *K. pneumoniae* resistant to ceftazidime (Poirel *et al.*, 2003). Lartigue and colleagues described the ability of IS*Ecp1B* to mobilise and express the β-lactamase gene, $bla_{\text{CTX-M}}$ from a transposon, which was located on a chromosome of *Kluyvera* ascorbata and moved to a plasmid (Lartigue *et al.*, 2006).

1.7.6.1 Integrative and Conjugative Elements (ICE)

Integrative and conjugative elements (ICE) are mobile genetic elements known as self-transmissible and found in Gram-positive and negative bacteria. ICE can be transferred from one strain to another by conjugation and lateral gene transfer. Like plasmids and phages, ICEs compromise of three modules divided according to functions responsible for maintenance, dissemination and regulation. ICEs maintain their virtual inheritance by integrating into a replicon of the host either plasmid or chromosome by means of gene encoding a recombinase called Int that catalyze *attP* on the ICE and a target sequence

attB on the chromosome. ICE, on the circular form, can be integrated into the chromosome by recombination between *attP* and *attB*, creating two ICE chromosome junction sequences, *aatL* and *attR*. ICEs are excised by excisionases called Xis and require the presence of *attL* and *attR* to perform excision. Dissemination of single stranded DNA of ICE is carried out by conjugation, genes specific for synthesis of mating machinery to enable the initiation between donor and recipient cell to deliver DNA to the recipient cell. (Wozniak & Waldor, 2010; Burrus & Waldor, 2004).

1.8 Objectives of study

Libya is located in North Africa bordered by the Mediterranean Sea from the north, Egypt from the east, Sudan from the southern east, Chad and Niger from the south and Algeria and Tunisia from the west. Libya is considered a very rich country; it has one of the most important resources worldwide, oil. Compared with other Arabic, European and Asian countries, Libya should have been one of the best countries in terms of development, infrastructure, investments and education; however, the last 40 years obfuscation and perfidy have retarded this potential.

Given that there is little information known on antibiotic resistance in Gram-negative bacteria in Libya and North Africa, I took the opportunity to investigate the mechanisms of antibiotic resistance in Gram-negative bacterial isolates collected from clinical, non-clinical and environmental settings in Tripoli and Benghazi, Libya.

The study focuses on the characterisation of antibiotic resistance mechanisms of isolates collected from patients admitted to different wards, in particular Intensive Care Units (ICUs). Moreover this study investigates the spread of MDR bacteria in the hospital environment to understand the occurrence of outbreaks within an individual hospital or among different hospitals. The emergence of MDR bacteria in non-clinical samples was also investigated in this study in order to associate the spread of nosocomial pathogens among

patients, within the hospital and outside the clinical settings. This is the first molecular study conducted on antibiotic resistance on bacterial strains isolated from Libya and will hopefully provide a useful insight on the problem of antibiotic resistance in Libya and in general Arabia.

Chapter Two

Methods and Materials

2.1 Bacterial collection.

Isolates used in this study were collected randomly from Tripoli and Benghazi from hospitals and environment outside the hospital during 2008-2009. Collection of the clinical samples included specimens from inpatients; outpatients and the hospital environment. Environmental swabs were collected from Tripoli and Benghazi streets, cafes etc. whereas the hospital environment samples refer to swabs collected from hospital floors, corners, toilets, walls, bedsides, sinks, curtains, trolleys, gauze containers, work tables and medical devices such as; mechanical ventilators, oxygen cylinders, baby incubators, nebulizers, anaesthesia, hypolizer, suction machine, tip of catheter. Bacterial isolates cultured from the swabs were identified by the use of Phoenix (Becton and Dickinson, USA). *E. coli* topo10 kit (Stratagene, Amsterdam, the Netherlands) was used in the cloning experiments. The swabs were transferred to the lab in transferring charcoal media (Technical Service Consultants Ltd, Heywood, UK).

2.1.1 Ethical considerations

The limited amount of information required for each specimen was such that ethical approval was not considered necessary and because there is no ethical board in Libyan hospitals.

2.2 Safety considerations

Regulations and safety were undertaken according to the Ionising Radiation Regulations, 1999.

2.3 Bacterial strains used

The following bacterial strains were used in cloning experiments

Strain	Genotype	Reference/Source
DH5α-T1^R	F$^-$ϕ80*lac*ZΔM15Δ(*lac*ZYA-*arg*F)U19 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A19 *rel*A1 *ton*A	**Invitrogen Ltd**
***E. coli* J53**	-	**Nordmann *et al.*, 2008**
***E. coli* GFP**	A modified *Escherichia coli* HB101 (UAB190) was used as the recipient strain [rifampicin and aminoglycoside resistant and green fluorescent protein (GFP) producing].	**Mata *et al.*, 2011**
***Pseudomonas aeruginosa* PA01**	-	**Barkay *et al.*, 1993**

2.4 Chemicals, reagents, and Radioactive labels.

Chemicals were purchased from BDH Chemicals Ltd and Sigma. Media constituents were obtained from either Oxoid laboratories or Fisher Scientific

laboratories. Radiolabelled Phosphorus ^{32}P was supplied from PerkinElmer, Boston, MA02118, United State of America (USA), 800-762-4000, Random primer labelling kits were supplied from Agilent Stratagene products, USA. PCR Gel extraction kits and plasmid miniprep purification kits were supplied from QIAGEN GmbH, D-40724 Hilden, Lambda Ladder PFGE Marker was obtained from New England Biolabs.Inc. Digestive enzymes; *Xba*I, *S*1 and *Spe*1 were purchased from Fermentas Life Sciences company. PCR Master Mix was supplied from Thermo Fisher Scientific ABgene House, Blenheim Road, Epsom, Surrey, UK.

2.5 Growth Media.

2.5.1 Luria Bertani Broth

L.B. broth was made up according to the manufacturer's instructions (Fisher Scientific Ltd).

2.5.2 Luria Bertani Agar

L.B. Agar was made following the manufacturer's instructions (Fisher Scientific Ltd).

2.5.3 Mueller-Hinton Agar

MHA was supplied by Oxoid Ltd plate poured ready to use in Etest experiments

2.5.4 MacConkey Agar No.3

MA no.3 was used to distinguish phonotypically between *K. pneumoniae* and *E. coli* in conjugation experiments, the medium was made up according to the manufacturer's instructions (Oxoid Ltd).

2.5.5 MacConkey Agar

MA was purchased from Oxoid Ltd plate poured ready to use.

2.5.6 MacConkey Agar for isolation of ESBL/MBL positive isolates

MA was made up and supplemented with 10mg/l of ceftazidime to be used as selective media; preparation of media was carried out according to the manufacturer's instructions (Oxoid Ltd).

2.5.7 S.O.C Medium

This was used as part of the TOPO10 cloning kit purchased from Invitrogen, Life Technologies, Carlsbad, California, USA.

2.6 Sterilisation of Media.

Media was sterilised by autoclaving at 0.75kg cm^{-2} for 20min at 121°C.

2.7 Isolation of environmental strains

Swabs collected from non-clinical settings and the environment outside the hospitals were cultured on MacConkey agar supplemented with 10mg/l of ceftazidime to select for isolates resistant to third generation cephalosporins.

Pure cultures were obtained by sub-culturing mixed cultures from the primary MA plates on a new selective MA plates supplemented with the same concentration of antibiotic.

2.8 Etest experiments

Etest strips containing imipenem (IP) and EDTA as MBL inhibitor (IPI) were purchased from. (BioMérieux, Paris, France). They were used to detect the occurrence of metallo-β-lactamases (MBLs) in carbapenem resistant isolates.

2.9 Antimicrobial Susceptibility Testing and MIC determination

Antibiotic resistance profile tests and minimum inhibitory concentration (MIC) determination for clinical, non-clinical and environmental isolates were performed according to the Clinical Laboratory standards Institute (CLSI) by the use of Phoenix 100 (Becton-Dickinson, Oxford, UK). MIC_{50} and MIC_{90} were defined as the minimal concentration that inhibited 50% and 90% of bacterial growth (Hsu *et al.*, 2011).

2.10 Phenotypic and Genotypic Detection of ESBLs

2.10.1 Amplification of DNA sequences using the Polymerase Chain Reaction (PCR)

2.10.1.1 Amplification of *bla*$_{CTX-M}$ type ESBLs

K. pneumoniae and *E. coli* isolates were screened for the occurrence of *bla*$_{CTX-M}$ type ESBLs that belongs to the phylogenetic groups, 1, 2, 8, 9 and 26

using multiplex PCR primers (Table 2.1) targeting a unique region in each group. The PCR experiments were performed using a set of specific primers and PCR conditions as described by Woodford and co-workers in 2006, the PCR products were then run on 1% (w/v) agarose gel to study their number and size in accordance to each phylogenetic CTX-M group (Woodford *et al.*, 2006). Some of the PCR products were selected to represent the source of samples from Tripoli and Benghazi, the PCR products were then cut of the gel and purified using PCR purification kit (QIAGEN GmbH, D-40724 Hilden), the purified PCR products were sequenced by an automated sequencer (377, ABI, Perkin-Elmer, CT) using the same amplification primers for each group of CTX-M family.

The reaction conditions used in the Thermal Cycler were as follows:

94°C for 5min	] 1 cycle
94°C for 25s	┐
52°C for 40s	| 30 cycles
72°C for 50s	┘
72°C for 6min	] 1 cycle

Table 2.1 Multiplex PCR for CTX-M- groups 1,2,8,9 and 26

CTX-M group	DNA sequence	Gene size
Group 1		
CTX-M-1 F	5-AAA AAT CAC TGC GCC AGTTC-3	415
CTX-M-1 R	5-AGC TTA TTC ATC GCC ACG TT	
Group 2		
CTX-M-2 F	5-CGACGCTAC CCCTGC TAT T-3	552
CTX-M-2 R	5-CCAGCGTCAGATTTT TCA GG-3	
Group 8		
CTX-M-8 F	5-TCG CGT TAA GCG GAT GAT GC-3	666
CTX-M-8 R	5-AAC CCA CGA TGT GGG TAG C-3	
Group 9		
CTX-M-9 F	5-CAA AGA GAG TGC AACGGA TG-3	205
CTX-M-9 R	5-ATT GGA AAG CGT TCA TCA CC-3	
Group 26		
CTX-M-26 F	5-GCA CGA TGA CAT TCG GG-3	327
CTX-M-26 R	5-AAC CCA CGA TGT GGG TAG C-3	

2.10.1.2 Detection of *bla*$_{CTX-M}$ group 1 and IS*Ecp1* genes

E. coli and *K. pneumoniae* isolates positive for CTX-M group 1 were subjected to PCR experiments to examine the incidence of *bla*$_{CTX-M-15}$ encoding genes and the insertion sequence IS*Ecp1* gene that is located immediately upstream of the β-lactamase gene. Specific primers were designed to read and amplify the *bla*$_{CTX-M}$ group 1 alone and in association with the IS*Ecp1* (see appendix Table A.2), PCR conditions used were as follows; 1 cycle of heating at 94°C for 5min followed by 30 cycles of heating at 94°C for 25s, 52°C for 40s and 72°C for 1min, the reaction ended with 1 cycle of heating at 72°C for 6 min, for amplification of *bla*$_{CTX-M}$ group 1. The same PCR conditions were used to detect *bla*$_{CTX-M}$ group 1 in association with IS*Ecp1* with extended annealing time to 90s. Positive controls were not used as some PCR products were sequenced. When required, new primers were designed using primer designer version 1.01, scientific and educational software.

2.10.1.3 Amplification of *bla*$_{TEM}$ and *bla*$_{SHV}$, *bla*AmpC, class 1 integrons and transposons

K. pneumoniae and *E. coli* isolates that were confirmed for ESBLs production were further examined for the occurrence of TEM, SHV, *bla*$_{ampC}$, class 1 integrons transposons Tn*402* and Tn*21* genes by PCR using specific primers (see appendix Table A.2). The same conditions applied for *bla*$_{CTX-M-15}$

amplification were used in these experiments. The alleles were cut, purified and sequenced as previously described.

2.10.2 Phenotypic detection of MBLs

Carbapenem susceptibility of the positive isolates to MBLs was performed using Etest strips (AB BioMerieux, La Plane, France) and the results were interpreted in accordance with the manufacturer's guidelines. PCR was also conducted to study the occurrence of $bla_{\text{SPM-1}}$, bla_{VIM}, bla_{GIM}, $bla_{\text{NDM-1}}$, bla_{IMP}, $bla_{\text{SIM-1}}$, $bla_{\text{KHM-1}}$, $bla_{\text{AIM-1}}$and $bla_{\text{DIM-1}}$. The PCR conditions used to amplify class 1 integron(s) were the same as described in section 2.9.1.1 and for primers used (see appendix Table A.3) with a slight modification where the annealing temperature in these conditions was 53°C and the elongation temperature was 68°C. All the PCR products were run on 1% (w/v) of agarose gel and the gels were then photographed. The resultant PCR products were purified from the agarose gel and sequenced using an automated sequencer (377, AB, Perkin-Elmer, CT).

2.11 Detection of $bla_{\text{OXA-48}}$ and IS*1999*

PCR experiments were performed on *K. pneumoniae* isolates to detect the occurrence of $bla_{\text{OXA-48}}$ and the insertion sequence IS*1999* using specific primers targeting the forward and reverse side of both genes (Table 2.2). PCR products were run in 1% of (w/v) agarose gel in TBE buffer, the electrophoresed gels were photographed.

Table 2.2 Oligonucleotide sequences to detect *bla*$_{OXA-48}$ and IS*1999*

Gene target	Primer name	Sequence	Reference
OXA-48 A	*bla*OXA-48	5' TTG GTG GCA TCG ATT ATC GG '3	Poirel, L. *et al.*, 2004
OXA-48 B	*bla*OXA-48	5' GAG CAC TTC TTT TGT GAT GGC '3	Poirel, L. *et al.*, 2004
IS1999 A	*IS*1999	5' CAG CAA TTC TTT CTC CGT G '3	Poirel, L. *et al.*, 2004
IS1999 B	*IS*1999	5' CAA GCA CAA CAT CAA GCG C '3	Poirel, L. *et al.*, 2004

2.12 Random amplified polymorphic DNA (RAPD) typing

2.12.1 RAPD DNA extraction by Chelex prep

It is a PCR-based technique used to differentiate between bacterial species by using short primers (Table 2.3) to anneal various locations of the bacterial DNA. A 24 h growth of *K. pneumoniae* isolates plated on MacConkey agar were used without selection and bacterial colonies were picked from the plate by inserting a sterile 200 µl plastic pipette tip into the colonies and dipped into 50 µl of an autoclaved solution of 5% Chelex® 100 resin (Biorad, Hertfordshire, UK).

To resuspend the mixture in the tube, it was agitated briefly. DNA extraction was carried out twice by heating the mixture to 89°C for 5 minutes on a heated block; the samples were immediately transferred to a 4°C chilled block. To sediment the chelex resin and cell debris, the samples were centrifuged for 5 minutes at 13.000 g and 2 µl of the clear supernatant was used as a template DNA to run the PCR.

2.12.2 Random amplified polymorphic DNA (RAPD-PCR)

RAPD-PCR fingerprinting was performed on 80 isolates of *K. pneumoniae* (12 isolates per reaction) as described by Mahenthiralingam *et al.*, 1996. RAPD-PCR was conducted using primer 272 (table2.3) for all reactions. For confirmatory purposes; RAPD-PCR using primer 270 (table 2.3) was carried out on subsets of *K. pneumoniae* isolates. PCR master mix was prepared prior to each experiment (1X PCR buffer, 1X Q-solution, 3mM $MgCl_2$, 200 µM dNTPs mixture, 1.6 µM RAPD primer, 1 U of Taq polymerase and 2µl of Chelex template DNA. PCR was run on a Flexigene Thermal Cycler (Techne Ltd., Newcastle, UK) using the following PCR conditions; 5 minutes of heating at 94°C, 4 cycles at 36°C for 5minutes, 72°C for 5 minutes and 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes. The last step was 72°C for 10 minutes.

2.12.3 DNA profile analysis by Agilent Bioanalyzer

1 µl of each PCR product was run for 20 minutes on an Agilent Bioanalyzer 2100 (Agilent Technologies UK Limited, Cheshire, UK) and a DNA 7500 chip contained 13 wells was used; 12 wells filled with samples, 1 µl in each and one filled with the ladder marker. The wells were also loaded with DNA gel matrix and an internal marker according to the manufacturer's protocol. After each run the results were saved as csv files.

2.12.4 GelCompar analysis

All csv-files were converted to a format compatible to GelCompar, similarities between fingerprints were calculated to the Pearson coefficient and unweighted pair group method with arithmetic means (UPGMA) was used to construct the dendrogram.

2.13 Multilocus sequence typing (MLST)

A subset of *K. pneumoniae* isolates were selected according to the RAPD-PCR fingerprinting results and MLST analysis was carried out as described by Diancourt *et al.*, 2005 and the MLST website (http:/ /www .pasteur .fr /recherché/genopole/PF8/mlst/Kpneumoniae.html) developed by Jolley *et al.*, 2004. Specific primers were used (Table 2.3) to amplify fragments of the following 7 housekeeping genes; β-subunit of RNA polymerase (*rpoB*), glyceraldehyde 3-phosphate dehydrogenase (*gapA*), malate dehydrogenase

(*mdh*), phosphoglucose isomerase (*pgi*), phosphorine E (*phoE*), translation initiation factor 2 (*nfB*) and periplasmic energy transducer (*tonB*).

The PCR conditions used for *rpoB*, *mdh*, *pgi*, *phoE* and *nfB* were as follows

94°C for 5min	**] 1 cycle**
94°C for 5min	┐
50°C for 30s	**| 30 cycles**
68°C for 1min	┘
68°C for 10min	**] 1 cycle**

The same PCR conditions were used for *gapA* and *tnoB* apart of the annealing temperature which was 50°C for *gapA* and 60°C for *tnoB*. All PCR products were run on 1% (w/v) Agarose gel and the gels were photographed. All PCR products were sequenced using an automated sequencer (377, ABI, Perkin-Elmer, CT) and the same amplification primers apart of *inf* forward primer which was replaced with (5'- ACT AAG GTT GCC TCC GGC GAA GC -3') and *pgi* primers were replaced with pgi2F; (5'- CTG CTG GCG CTG ATC GGC AT -3') and pgi 2R (5'- TTA TAG CGG TTA ATC AGG CCG T-3').

Table 2.3. Oligonucleotides used for PCR amplification and DNA sequencing

Gene target	Primer name	Primer sequence	Reference
ropB	*ropB* F	5'-GGCGAAATGGCWGAGAACCA-3'	Diancourt *et al.*, 2005
ropB	*ropB* R	5'-GAGTCTTCGAAGTTGTAACC-3'	Diancourt *et al.*, 2005
gapA	*gapA* F	5'-TGAAATATGACTCCACTCACGG-3'	Diancourt *et al.*, 2005
gapA	*gapA* R	5'-CTTCAGAAGCGGCTTTGATGGCTT-3'	Diancourt *et al.*, 2005
Mdh	*Mdh* F	5'-CCCAACTCGCTTCAGGTTCAG-3'	Diancourt *et al.*, 2005
Mdh	*Mdh* R	5'-CCGTTTTTCCCCAGCAGCAG-3'	Diancourt *et al.*, 2005
Pgi	*pgi* F	5'-GAGAAAAACCTGCCTGTACTGCTGGC-3'	Diancourt *et al.*, 2005
Pgi	*pgi* R	5'-CGCGCCACGCTTTATAGCGGTTAAT-3'	Diancourt *et al.*, 2005
phoE	*phoE* F	5'-ACCTACCGCAACACCGACTTCTTCGG-3'	Diancourt *et al.*, 2005
phoE	*phoE* R	5'-TGATCAGAACTGGTAGGTGAT-3'	Diancourt *et al.*, 2005
infB	*infB* F	5'-CTCGCTGCTGGACTATATTCG-3'	Diancourt *et al.*, 2005
infB	*infB* R	5'-CGCTTTCAGCTCAAGAACTTC-3'	Diancourt *et al.*, 2005
tnoB	*tnoB* F	5'-CTTTATACCTCGGTACATCAGGTT-3'	Diancourt *et al.*, 2005
tnoB	*tnoB* R	5'-ATTCGCCGGCTGRGCRGAGAG-3'	Diancourt *et al.*, 2005
Integrase gene	VAF	5' GCCTGTTCGGTTCGTAAGCT 3'	
PAPD-PCR Primer	272	5'- AGC GGG CCA A -3'	Mahenthiralingam *et al.*, 1996
PAPD-PCR Primer	270	5'- TGC GCG CGG G -3'	Mahenthiralingam *et al.*, 1996

2.14 Plasmid identification

Plasmids are circular extra-chromosomal DNA; they are known to play a role in changing the diversity of the bacterial genome by acquiring or losing genes such as antibiotic resistance genes, subsequently contribute to the movement and transfer of resistance mechanisms from bacteria to bacteria by means of horizontal gene transfer (Carattoli, A. *et al.*, 2005). PCR-based replicons typing was performed to identify plasmids contributed to the dissemination of ESBL and MBL genes among Libyan isolates using 5 multiplex and 3 simplex PCR experiments as described by Carattoli, A. *et al.*, 2005. This procedure is used to identify the major plasmids that are known as incompatible plasmids by recognizing FIA, FIB, FIC, HI1, HI2, I1, Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA using eighteen pairs of primers designed to be conducted on 8 PCRs. The 5 multiplex PCRs are designed to recognize three plasmids for each reaction (see appendix Table A.4). Positive controls were used to comaper size of plasmids. The PCR conditions used to detect all plasmids apart of F simplex were as follows

94°C for 5min	**] 1 cycle**
94°C for 1min	┐
60°C for 30s	**| 30 cycles**
72°C for 1min	┘
72°C for 5min	**] 1 cycle**

Whereas the conditions of F simplex PCR were almost the same with only one difference as the annealing temperature was changed to 52°C.

2.15 Transconjugation experiments

Conjugation experiments were carried using *E. coli* J53 and GFP as recipients. Fresh colonies of parents and recipients were grown separately on LB broth media (Fisher Scientific, USA Products) in 50 ml Falcon tubes and incubated overnight at 37°C for 18 h. Each isolate of parents was mated with *E. coli* J53 or GFP *E. coli* in aliquots of 1:1 in a fresh LB broth media and incubated overnight at 37°C in shaking incubator. Transconjugants were selected by culturing 100 µl of each mating mixture on LB medium (Fisher Scientific, USA Products) supplemented with 200 µl/ml of sodium azide and 10 mg/l of ceftazidime. Parents that were mated with GFP *E. coli*, the selection was performed on L.B agar supplemented with 50mg/l of rifampicin and 10mg/l of ceftazidime. The plates were subsequently incubated overnight at 37°C for 18 h. Pure colonies of *E. coli* from each plate were picked and transferred to a fresh LB broth media supplemented with 200 µl/ml of sodium azide and 10 mg/l of ceftazidime for *E. coli* J53 transconjugants and with 50mg/l of rifampicin and 10mg/l of ceftazidime for GFP *E. coli* transconjugants. The transconjugants were then plated on LB media supplemented with the same concentrations of antibiotics used for parents and incubated overnight at 37°C for 18 h. The transconjugants that were grown on LB media were stored at -80 °C for further investigation. PCR experiments were performed on

transconjugants targeting *bla*$_{CTX-M}$ group 1 encoding genes and IS*Ecp1* for *K. pneumoniae* and *E. coli* using the forward and reverse primers from table (1).

2.16 Southern hybridisation

2.16.1 Characterization of chromosomally and plasmid mediated resistance genes.

2.16.1.1 Preparation of plugs of whole genomic bacteria DNA

Whole genomic DNA of the bacteria was used to prepare plugs to detect chromosomally and plasmid mediated genes. Bacterial cultures were grown overnight at 37°C. One loop of the fresh colonies of each isolate was suspended in 3 ml of normal saline and the optical density 600 (OD_{600}) of each isolate was measured and the formula (1.5/measured OD Multiplied by 300) to adjust the volume of cells to the equivalent of 300 µl in accordance to the OD_{600}. The suspended cells were then centrifuged at 13 Kg using mini-centrifuge (Minispin centrifuge, Hamburg, Germany) for 30s and the supernatant removed. Cells were then re-suspended in 300 µl of normal saline and transferred to a 50°C block heater. Cells were lysed by adding 2-3 drops of 25 mg/ml of lysozyme and a 2.5 % (w/v) of pre-warmed (50°C) low melting point agarose was quickly pipetted and gently mixed and quickly dispensed into PFGE plugs components and dried at room temperature for 30 min. 5 plugs of each set were then transferred into a 24 well plate and 2 ml of lysis buffer (10 mM Tris-Hcl, pH 7.2, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% N-Lauroylsarcosine) was added and supplemented with 80 µl of 25mg/l

of lysozyme. The plugs were then incubated at 37 °C for 1.5 hrs. The plugs at this stage were washed with 2 mls of 1X TE buffer (10 mM Tris-HCl, 50 mM EDTA pH 8.0; Bio-Rad) at 37°C for 30 mins. The TE was replaced with 2 mls of proteolysis buffer (100 mM EDTA pH 8.0, 0.2% sodium deoxycholate, 1% and N-Lauroylsarcosine; Bio-Rad) 20 µl of 10 mg/l of Proteinase K and incubated at 50°C for 18 hrs. After the proteolysis buffer was removed, the plugs were then washed five times with 1X TE buffer in shaking incubator at 37°C for 30 mins.

One plug of each set was transferred to a new 24 well plate and washed with 0.1 X TE buffer at 37 °C for 30 mins. The plugs were then washed twice with 2X S1 buffer at room temperature for 15 mins each. The 2X S1 buffer was removed and replaced with 1X S1 buffer and washing was performed at room temperature for 15 min. The S1 buffer was then removed and 1 µl of 20U of S1 endonuclease (Promega, USA) was added and the plugs were incubated at 37°C for 45 min. 100 µl of ES buffer (0.5 M EDTA, pH 8; 1% N-Lauroylsarcosine) was added to stop the digestion. PFGE gels were prepared; 0.88% (w/v) agarose in 0.5 X of TBE buffer (45mM Tris-base, 45 mM boric acid, 1 mM EDTA, pH 8.0; Bio-Rad) and 20 µl of ethidium bromide was added to stain the gels. Plugs were loaded into the gels and the gels were run in the PFGE tank (CHEF-DRIII system, Bio-Rad laboratories). Migration of DNA was performed at 9°C with initial switch time of 5 and final switch time of 45 for 20 hrs at 6 volts and 120 ° angle. Lambda Ladder was used as a DNA size marker.

2.16.2 Pulsed Field Gel Electrophoresis (PFGE) Typing

Plugs of the whole genomic DNA of the target bacterium was prepared as for *Spe*1 digests described by Patzer & Dzierzanowska, 2007. Each plug was washed with 0.01 x of TE buffer shaking at 37°C for 30 min, followed by washing twice with 300 µl of 2x of *Xba*1 fast digestive buffer (Fermentas, Sheriff Hutton Industrial Park, York, UK) for 15 min at room temperature and once with 300 µl 1x of Xba1 fast digestive buffer for 15 min. The DNA in plugs was then digested with 3.5 µl of Xba1 overnight at 37°C for *K. pneumoniae* and *E. coli*. The same steps were performed on plugs made of whole genomic DNA from *P. aeruginosa* but washed with Spe1 buffer and digested with 1 µl of *Spe*1 enzyme. Separation of *Xba*1 and *Spe*1 digested DNA was performed by using PFGE apparatus (CHEF-DRIII system, Bio-Rad laboratories) and DNA migration was conducted using the following conditions; initial switch time at 5s and final switch time at 45s, 6V/cm and 120° angle for 20h with cooling at 9°C, using TBE buffer (0.5x Tris borate, 0.5mM EDTA), Lambda ladder DNA was used as a marker to size DNA. The interpretation of similarities between bacterial species was performed as described by (Tenover *et al.*, 1995). The resultant PFGE Gels were photographed and dried overnight on a Whatman filter paper (15 cm * 15 cm) blotting paper, the gels were then re-hydrated, denatured using a denaturing buffer (0.5M NaOH, 1.5M NaCl) for 30 min at room temperature, neutralized using a neutralizing solution (0.5M Tris-HCl, pH 7.5, 1.5M NaCl) for 30 min

at room temperature. The gels were then transferred to a hybridization tube contains pre-hybridization solution at 65°C and probed with a ^{32}P radio-labelled CTX-M-15 template DNA and CTX-M-15/IS*Ecp1* for *K. pneumoniae* and *E. coli* and the *P. aeruginosa* PFGE gels were probed with a ^{32}P radio-labelled VIM-2 as described by Patzer *et al.*, 2009.

2.16.3 Colony Blotting.

Colony blotting experiments were carried out by using a modification of the procedure of (Ivanov & Gigova, 1989). MacConkey agar plates were spotted with the isolates of interest and incubated overnight at 37°C for 18 h. MacConkey agar plates with bacterial isolates were photographed using digital camera and then overlaid with a circular membrane (Hybond^{TM} , Amersham Pharmacia, UK), for at least 2 min, so the bacterial isolates will have been transferred to it. The membranes were then removed by a sterile forceps and placed colony side up on a presoaked 15cm^2 Whatman blotting paper (Whatman inc. Sigma-Aldrich, Sanford, UK) with 5% of SDS (sodium dodicyl sulphate) for 5 min at room temperature. The membranes were then carefully transferred to a 15cm^2 Whatman blotting paper to remove any excess moisture, the membranes were then placed colony side up on 15cm^2 Whatman blotting paper presoaked with denaturing solution (1.5 NaCl, 0.5 M NaOH) for 5 min.

The membranes were then carefully removed and dabbed dry on 15cm^2 Whatman blotting paper and transferred and floated colony side up in

neutralizing solution (157 g. Tris-HCl, 174 g. NaCl in 2L of H2O pH 7.5) for 5 min. The cellular debris was then carefully removed and washed with 6X SSC (6 ml of 20X SSC in 20 mls of demonized water) and dabbed dry. The membranes were then dried at 80°C for at least 3h to fix the DNA to the membrane filters. The membrane filters were then transferred to hybridization tube provided with hybridization solution (6X SSC, 0.1 % (W/V) polyvinylpyrrolidine (PVP), 1 ml of 0.5 % (W/V) SDS, 400 µl of 0.1% (W/V) ficoll, 400 µl of Milk and 300 µl of 150 $\mu g/ml^{-1}$ denatured spermatozoid DNA). The hybridization tube was then incubated at 65°C prior to probing with gene of interest.

2.16.4 In gel hybridization

The resultant PFGE gels were photographed and dried at 50°C for 18 hrs, the gels were then hybridized as follows; rehydrated in DNA free water for 30 mins at room temperature, the DNA in gel was denatured for 30 mins using denaturing solution (NaCl, 0.5 M NaOH) and neutralized by neutralizing solution (Tris-HCl, NaCl) for 30 mins. The gels were then transferred to hybridization tubes with pre-hybridization solution (6X SSC, 0.1% (W/V) polyvinylpyrrolidine (PVP), 1 ml of 0.5% (W/V) SDS, 400 µl of 0.1% (W/V) ficoll, 400 µl of Milk and 300 µl of 150 $\mu g/ml^{-1}$ denatured spermatozoid DNA) and incubated at 65°C overnight. The hybridized gels were subsequently probed. Gels were then washed twice, once with 2X SSC (Sodium Citrate), 0.1% (W/V) SDS and once with 0.1 X SSC, 0.1% (W/V)

SDS. The gels were then wraped in cling film and transferred to a cassette and a Hyperfilm^{TM} (Amersham, GE Healthcare, Life Sciences) was firmly pressed on the gel and frozen at -80°C for 18 hrs. Developer and fixer were used to detect the appearance of any radio labeled spot on the Hyperfilm.

2.16.5 Labelling DNA Probes

To produce high specific activity probes, labeled DNA was generated using random oligonucleotides, and anneal to specific sites on the DNA template. The Klenow will use the primer-template complex as a substrate and synthesize a new DNA by incorporating monophosphates at the free 3′-OH group. Radio-labeling is performed by exchanging the nonradioactive with the radioactive in the reaction mixture. The radio-labeled gene will then serve as a sensitive hybridization probe, it is used in southern and northern blots and in Situ hybridization techniques. The genes of interest ($bla_{CTX\text{-}M\text{-}15}$ alone and in association with IS*Ecp1*, $bla_{VIM\text{-}2}$, *tni*C, bla_{TEM}, bla_{SHV}, *ISCR2* and $bla_{TMB\text{-}1}$) were amplified by PCR using specific primers targeting the forward and reverse regions of the gene to be used as a template DNA to probe the hybridized membranes. 15 µl of the template DNA was mixed with 8 µl of DNA free water and 10 µl of random 9-mer primers (Agilent Technologies – Stratagene – USA Products) were added in a screw capped Eppendorf tube. The mixture was firstly boiled in a water bath for 5 min and immediately 10 µl of 5X dCTP buffer, 2,5 µl of the radioactive phosphorus ^{32}P and 1 µl of Exo(-) Klenow (Agilent Technologies – Stratagene – USA Products) were added to

the mixture and transferred to a jar made of lead and incubated at 37°C for 15 min to allow the production of the radio-labeled template DNA. The radio-labeled product was then pipetted into a silica gel column (Nick^{TM} columns Sephadix, G-50 DNA Grade, illustra, GE Healthcare, Life Science, UK). The column was then washed with 320 µl of washing buffer (0.1 M Tris-Hcl Buffer, PH 7.5) followed with 430 µl of the same washing buffer to an Eppendorf tube to elute the radio-labelled gene purified. The radio-labeled PCR product was then boiled in a water bath for 6 min to denature the double stranded template DNA, the probe was then added to the previously incubated membranes or gels (see sections 2.15.3 and 2.15.4) in the hybridization tube and incubated over night at 65°C.

2.17 Cloning Experiments

Cloning experiments were performed on an *A. xylosoxidans* isolate trying to obtain the full sequence of the new MBL gene. The cloning experiments were carried out by chemical transformation (Blue/White) screening test by using the plasmid vector (pCR®4-TOPO®) and *E. coli* 5DHα supplied by TOPO10 cloning kit supplied by (Invitrogen Ltd, Inchinnan Business Park, 3 Fountain Drive, Paisley, UK). The 3kb PCR products were amplified from the A. *xylosoxidans* and purified before using it in the cloning experiments. TOPO cloning reaction was performed by mixing the 3kb class 1 integron, salt solution (1.2 M NaCl 0.06 M MgCl2) and TOPO vector at room temperature and then kept on ice. To perform transformation, 2µl of the reaction was then transferred into a vial containing chemically-competent *E. coli* and incubated

on ice for 30 minutes. The *E. coli* was heat shocked at 42°C for 30s without shaking and immediately returned to ice and 250 µl of S.O.C broth medium then added and incubated at 37°C for 1h. A total of 50µl of the broth culture was streaked on L.B. Agar (Fisher Scientific, USA products) plates supplemented with 50 mg/l of kanamycin, X-galactose and isopropyl-β-D-thiogalactoside (IPTG) and then incubated at 37°C for 18h. The white colonies were picked up and grown overnight in L.B broth, the TOPO vector was then extracted from the cells by miniprep kit and sequenced using the primers M13 forward and reverse.

2.18 Purification of TMB-1

2.18.1 Expression

TMB-1 was purified directly from the *A. xylosoxidans* isolate grown overnight at 37°C in flasks containing 4x 50ml of Terrific broth (Sigma, St. Louis, MO, USA) supplemented with 50mcg/ml of kanamycin, the cultures were then incubated shaking at 37°C. Each flask was inoculated with 4x 1L of Terrific broth with 50ug/ml of kanamycin and flasks incubated at 37°C and 225 rpm. The production of the protein was induced by IPTG (final concentration 0.1mM) when $O.D_{600}$ is between 0.6-0.7. Cells were centrifuged at 7000 g for 10 min at 4°C. The expression of protein was confirmed using Sodium SDS-Page.

2.18.2 Periplasm isolation

To perform large scale protein preparations of periplasmic cellular extracts, it was necessary to treat cells with lysozyme. The methods used were that of Avison *et al.*, 2011 and Samuelsen *et al.*, 2008. The cell pellets were resuspended in buffer (50mM Tris-HCl, 100uM ZnCl2, 0.02% NaN3 pH 7.2). The lysozyme was then added to a concentration of 200μg/ml. The suspension was then incubated rotating at room temperature for 15-20 min. CaCl2 was then added to a concentration of 10mM, the suspension was then centrifuged at 9000xg or 18000 rpm for 20 min. at 4°C.

2.18.3 Purification of β-lactamase from crude periplasmic cell extract

The crude cell extract was loaded on to 50 ml Q-Sepharose column (Q-Sepharose HP column, Pharmacia, GE Healthcare, UK) that was previously pre-equilibrated with 100 ml of buffer (buffer (50mM Tris-HCl, 100uM ZnCl2, 0.02% NaN3 pH 7.2).The protein was then loaded and eluted using 400 ml NaCl gradient. The eluted fractions were collected and checked for β-lactamase activity using Nitrocefin. Purity of fractions that showed β-lactamase was performed on SDS-PAGE (2-14% NuPAGE Bis-Tris mini gels).

2.18.4 Gel-filtration

Column was pre-equilibrated with two column volume of washing buffer (see section 2.18.2), the protein was loaded through a super loop (flow 1ml/min) and then wash or elute the protein with (100-300) of washing buffer. Fraction were then collected and checked for β-lactamase using Nitrocefin, the active fractions were run on SDS-PAGE and stored at 4°C. TMB-1 was analysed using nitrocefin +/- EDTA and SDS-PAGE. TMB-1 was concentrated to 1.94mg/ml.

2.19 Kinetics assay:

Steady-state kinetics was performed at 25°C in a spectrophotometer (SpectramaxPlus, Molecular Devices) using 96 well plates (BD Falcon UV microplates, BD Biosciences, USA) (Samuelsen *et al.*, 2008). All substrates (ceftazidime, cefoxitin, cefuroxime, piperacillin, ampicillin, imipenem, meropenem and ertapenem) were tested as duplicates using 50mM HEPES pH 7.2, 100μM $ZnCl_2$, 0.02% NaN_3, and 0.1mg/ml bovine serum albumin (Sigma-Aldrich) as a buffer system. The kinetic data were analysed by non-linear regression (GraphPad Software, San Diego, CA).

Chapter Three

Characterization of Multi-drug resistant *Klebsiella pneumoniae* from Tripoli & Benghazi, Libya

3.1 Introduction

K. pneumoniae can be isolated from a variety of different sites, locations and environments such as, water and soil, or from hospitalised patients or from animals. Such variation in habitats provides *K. pneumoniae* with the opportunity to spread quickly and as a consequence it can cause infections (Podschun & Ullmann, 1998). Infections due to MDR strains of *K. pneumoniae* have been reported world-wide in neonatal wards, ICUs, paediatric hospitals (Podschun & Ullmann, 1998; Bagattini *et al.*, 2006), UTIs and lower respiratory tract infections (Gori *et al.*, 1996; Podschun & Ullmann, 1998; Cartelle *et al.*, 2004; Valverde *et al.*, 2008; Kiratisin, 2008). It is increasingly reported year on year (Grobner *et al.*, 2009; Lim *et al.*, 2009) and thus represents a major clinical threat particularly for immunocompromised patients (Oteob*et al.*, 2009).

The frequent use of extended-spectrum cephalosporins, particularly in ICUs is considered a leading factors contributing to epidemic and endemic outbreaks of nosocomial infection as a result of the emergence of MDR Gram-negative pathogens producing ESBLs (Gori *et al.*, 1996;Valverde *et al.*, 2008). ESBLs are the most prevalent enzymes produced by multi-resistant strains of *K. pneumoniae* and are capable of hydrolysing most β-lactams particularly third and fourth generations cephalosporins (Wei *et al.*, 2005).

Nosocomial infections caused by ESBLs producing *K. pneumoniae* have become a major problem in the United States, Europe, Asia (Livermore, 2009), Africa (Gori *et al.*, 1996), Brazil and Spain (Rodriguez-Bano *et al.*, 2010). ESBLs are often carried on plasmids of different sizes and types reflecting the frequency and epidemiology of these enzymes (Gori *et al.*, 1996). CTX-M-type ESBLs are encoded by genes carried on plasmids of different types such as; IncF1, IncFII, IncH12 and IncI which are classified as narrow host-range types of plasmids and known to mobilize $bla_{\text{CTX-M-15}}$ and IS*Ecp1*. Furthermore, IncN, IncP-1-[a], IncL,/M as well as Inc A/C are broad host-range plasmids and effective as transmissible elements and play important roles in the dissemination of $bla_{\text{CTX-M-15}}$ genes (Pitout, 2010; Carattoli, 2009). Such replicons can act as major vehicles for the horizontal transfer of genes responsible for antibiotic resistance that cause CAIs and HAIs (Colinon *et al.*, 2007). CTX-M type extended spectrum-β-lactamases are considered the most prevalent ESBLs among *E. coli* and *K. pneumoniae*. These enzymes have originally been derived from chromosomal β-lactamase from *Kluyvera* spp. (Dedeic-Ljubovic *et al.*, 2010).

$bla_{\text{CTX-M-15}}$ is one of the most important enzymes of the 120 variants of CTX-M type ESBLs found to date (http://www.lahey.org/Studies/other.asp#table1) and was first discovered in India, France and Japan in the 1980s and recently worldwide (Yu *et al.*, 2004; Lartigue *et al.*, 2007; Touati *et al.*, 2006; Abbassi *et al.*, 2008; Gonullu *et al.*, 2008; Walsh, 2006). $bla_{\text{CTX-M-15}}$ has a broader

substrate profile than many other CTX-Ms due to mutations around the active site (Pitout, 2010). Several reports have mentioned the occurrence of $bla_{\text{CTX-M-15}}$ associated with the insertion sequence IS*Ecp1* located upstream of the CTX-M gene in *E. coli* and *K. pneumoniae* from Nigeria, Norway, Tunisia, UK and France (Touati *et al.*, 2006; Abbassi *et al.*, 2008; Eckert *et al.*, 2006; Kiratisin *et al.*, 2008; Ben Salma *et al.*, 2011; Younes *et al.*, 2011).

$bla_{\text{CTX-M-15}}$ has also been detected in the Mediterranean area, the Middle East and the Arab Gulf region. The CTX-M-15 gene has been found in clinical isolates of *E. coli* from Cairo, Egypt and associated with the insertion sequence, IS*Ecp1* (Khalaf *et al.*, 2008). *K. pneumoniae* and *E. coli* harbouring $bla_{\text{CTX-M-15}}$ were found disseminated in neonatal wards and ICUs in Saudi Arabia (Al-agamy *et al.*, 2009), Algeria (Ramadani-Bouguessa *et al.*, 2006) and Kuwait (Dashti *et al.*, 2010). Similarly, $bla_{\text{CTX-M-15}}$ was found plasmid mediated in clinical isolates of *E. coli* collected from Egypt (Mohamed Al-Agamy *et al.*, 2006).

This chapter describes the emergence of MDR *K. pneumoniae* isolates from clinical settings (patients and hospital environment) and non-hospital environmental isolates. The phenotypic characteristics and the antibiotic resistance profile of 80 *K. pneumoniae* isolates are determined and discussed.

3.2 Results

3.2.1 Antimicrobial susceptibility testing

K. pneumoniae isolates collected are listed in table 3.1. The MIC_{50}, MIC_{90} and MIC ranges of 80 isolates of clinical, hospital environment and non-hospital environment *K. pneumoniae* are presented in table 3.2. These results show that MIC_{50} and MIC_{90} of ceftazidime was higher that of cefotaxime. The highest MIC_{50} and MIC_{90} were observed for piperacillin/tazobactam whereas the lowest was for the carbapenems; imipenem and meropenem. The highest level of resistance (95 %) has been observed against the antibiotics piperacillin and ampicillin. Thirty five out of eighty (43.75 %) of *K. pneumoniae* exhibited resistance against piperacillin/tazobactam. The results also showed that 52/80 (65%) showed resistance to amoxicillin/clavulanic acid combinations and 3 others showed intermediate resistance to amoxicillin/clavulanic acid combinations. Resistance to aminoglycosides varied; 2/80 (2.5 %) showed resistance to amikacin, whereas 49/80 (61 %) were resistant to gentamicin. 33/80 (41 %) were resistant to ciprofloxacin and another one was intermediate. Fifty six out of 80 isolates (70 %) were resistant to cefuroxime whereas 48/80 (60 %) and 49/80 (61 %) displayed resistance to ceftazidime and cefotaxime, respectively; and 46/80 (57.5 %) were indicated by Phoenix as ESBL positive. Of those that are ESBL positive, resistance was observed for amoxicillin/clavulanate and piperacillin/tazobactam with 38/46 (82.6 %) and 29/46 (63%), respectively.

3.2.2 Genotypic detection of *bla*$_{OXA-48}$ and ESBLs

3.2.2.1 The prevalence of CTX-M groups 1, 2, 8, 9 and 26

The results of detection of the occurrence of CTX-M groups 1, 2, 8, 9 and 26 are shown in Figure 3.1. This experiment was based on the amplification of part of the targeted gene of each group of CTX-M-type ESBLs. 50/80 (62.5 %) of the *K. pneumoniae* isolates demonstrated the presence of CTX-M group 1. None of the isolates produced any PCR products when specific primers were used for CTX-M groups 2, 8, 9 and 26.

Table3.1 Dissemination of *K. pneumoniae* in Tripoli and Benghazi, RAPD clusters, MLST and *bla*$_{CTX-M}$ group1 results

Location	Number of isolates	RAPD clusters	MLST	*bla*CTX-M group 1
Al-Jamhoriya hospital Benghazi	*n=21*	1,2 & 5	ST147, ST101	16
Al-Jala hospital of Benghazi	*n=8*	1,2 & 6	ST101	5
7th of October hospital	*n=8*	2	ST15 (*n=2*)	7
Kwaifia hospital Benghazi	*n=5*	1,2 & 6	ST29	2
Benghazi Pediatric hospital	*n=6*	1 & 2	0	5
Tripoli medical centre	*n=1*	5	0	0
Tripoli Milatiry hosital	*n=2*	1, 2 & 4	0	1
Tripoli maternity hosital	*n=11*	1 & 2	ST70	6
Burn and plastic surgery centre of Tripoli	*n=12*	1,2,4 & 6	ST111, ST15	7
Tripoli pediatric hospital	*n=1*	1	0	0
Benghazi lake	*n=1*	1	0	0
Syria area Benghazi	*n=2*	6	ST506, ST486	1
Keesh area Bemghazi	*n=1*	6	0	0
Dollar area Benghazi	*n=1*	2	ST511	1

Table 3.2 MIC_{50} and MIC_{90} of *K. pneumoniae*

Antibiotic	MIC_{50}	MIC_{90}	Range mg/l
Ceftazidime	16	32	4 - 32
Cefotaxime	8	64	2 - 64
Imipenem	0.5	1	0.125 - 1
Meropenem	0.5	1	0.125 – 1
Aztreonam	16	32	8 – 32
Piperacillin/Tazobactam	32	128	4 – 128
Ciprofloxacin	4	8	0.5 – 8
Ampicillin	16	64	4 – 64
Gentamicin	8	16	2 – 16

3.2.2.2 Detection of CTX-M-15 genes and IS*Ecp1*

The results of detection of the incidence of $bla_{CTX\text{-}M\text{-}15}$ in a subset of 11 *K. pneumoniae* isolates (AES64, AES178, AES261, AES268, AES273, AES274, AES280, AES970, AES973, AES984 and AES1001) are shown in Figure 3.2. Sequencing of some PCR products showed the occurrence of $bla_{CTX\text{-}M\text{-}15}$ genes in Libyan *K. pneumoniae* (B.18- B31). The results suggest that $bla_{CTX\text{-}M\text{-}15}$ is the gene responsible for the production of ESBL and mediates extended spectrum cephalosporin resistance in some Libyan *K. pneumoniae*. Amplification of $bla_{CTX\text{-}M\text{-}15}$ genes in association with the insertion sequence

IS*Ecp1* are illustrated in Figure 3.3. In addition Figure 3.4 show the association between bla_{CTX-M} group1 gene with the insertion sequence IS*Ecp1* and the differences that are noticed due to the presence/absence of an intact copy of the insertion sequence in some of these isolates. The insertion sequence IS*Ecp1* is the promoter for the movement and expression of the cefotaximase encoding gene and it is more often than not located upstream of the β-lactamase gene (Poirel *et al.*, 2003). PCR products obtained by amplification of $bla_{CTX-M-15}$ genes and IS*Ecp1* from 11 of *K. pneumoniae* isolates using two forward primers (ISEcpu1 and ISEcpu2) targeting two different sites on the insertion sequence) and the standard reverse primer (CTX-M-15 R) produced different sized products.

The results suggest the occurrence of a deletion event in IS*Ecp1* in some of the isolates, and PCR using different primers failed to amplify the insertion sequence and $bla_{CTX-M-15}$ and consequently appeared negative (Figure 3.4). The deletion was confirmed by using the forward primer ISEcup2 with the reverse primer for the $bla_{CTX-M-15}$ gene. On the same isolates, the results of the amplification of $bla_{CTX-M-15}$ gene and IS*Ecp1* using ISEcup1 forward primer with CTX-M-15 reverse primer were able to prove the occurrence of $bla_{CTX-M-15}$ in association with partial copy of IS*Ecp1* (Figures 3.3 & 3.5).

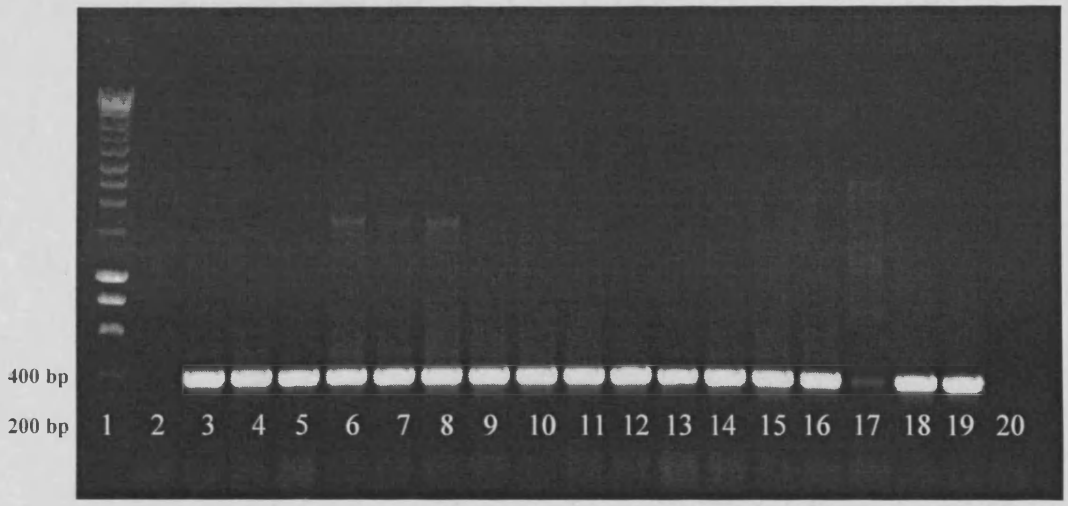

Figure 3.1 Multiplex PCR experiment to detect the incidence of CTX-M type ESBLs groups 1, 2, 8, 9 and 26. Lane1: Marker. Lane2: *K. pneumoniae* isolate AES7. Lane3: AES8. Lane4: AES48. Lane5: AES53. Lane6: AES59. Lane7: AES64. Lane8: AES66. Lane9: AES67. Lane10: AES68. Lane11: AES73. Lane12: AES74. Lane13: AES85. Lane14: AES103. Lane15: AES104. Lane16: AES135. Lane17: AES136. Lane18: AES140. Lane19: AES141. Lane20: AES145.

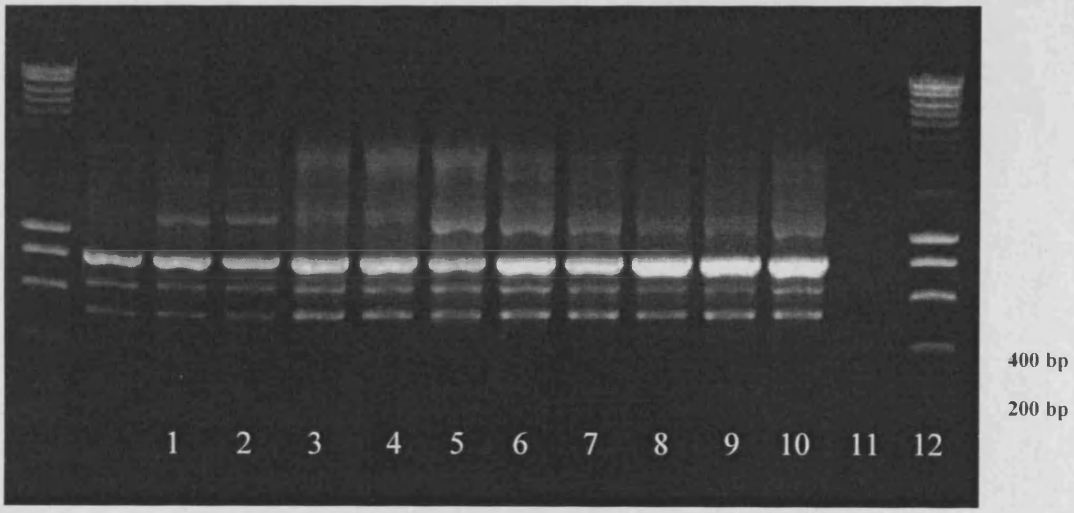

Figure 3.2 PCR experiment to detect the incidence of $bla_{\text{CTX-M-15}}$ in *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES64. Lane3: AES178. Lane4: AES261. Lane5: AES268. Lane6: AES273. Lane7: AES274. Lane8: AES280. Lane9: AES970. Lane10: AES973. Lane11: AES984. Lane12: AES1001. Lane13: Negative control. Lane14: Marker

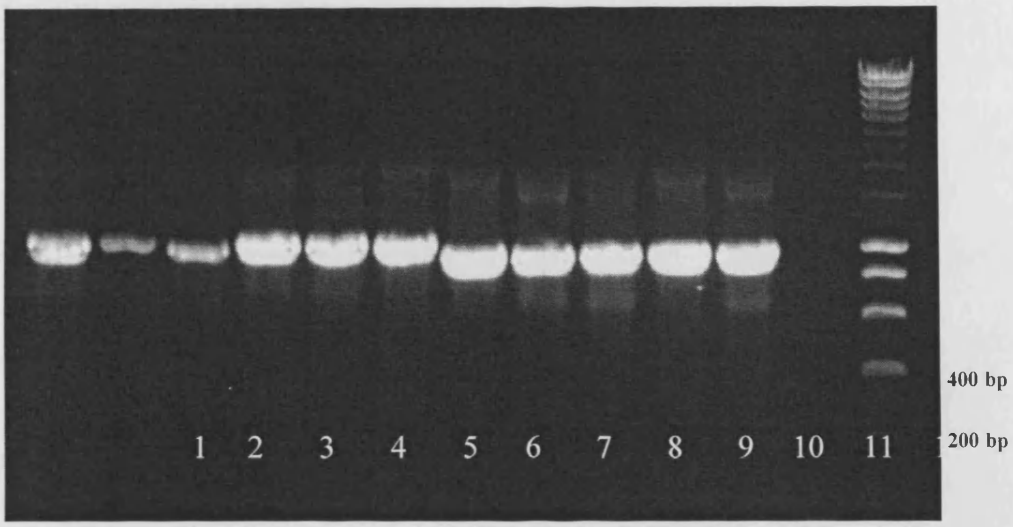

Figure 3.3 PCR experiment to detect the incidence of bla_{CTX-M} group1 in association with IS*Ecp1* in *K. pneumoniae* isolates. Lane1: AES64. Lane2: AES178. Lane3: AES261. Lane4: AES268. Lane5: AES273. Lane6: AES274. Lane7: AES280. Lane8: AES970. Lane9: AES973. Lane10: AES984. Lane11: AES1001. Lane12: Negative control. Lane13: Marker

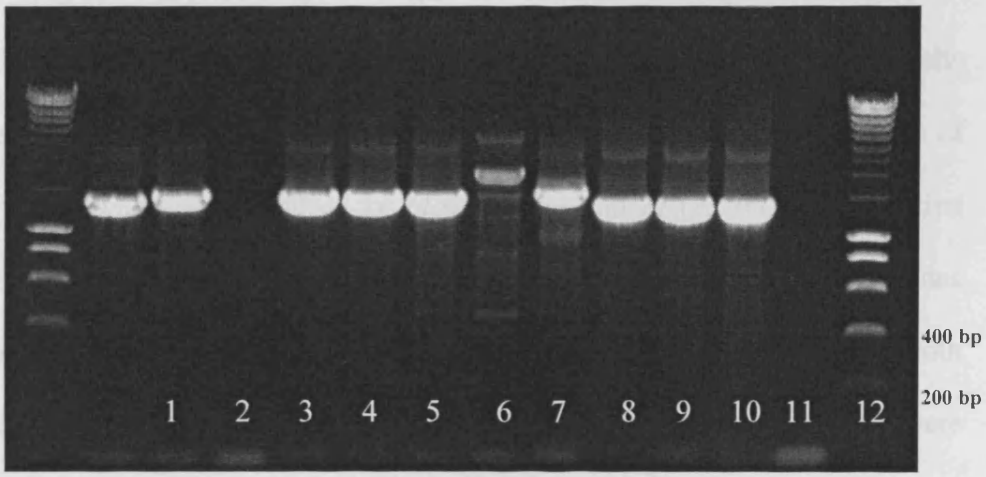

Figure 3.4 PCR experiment to detect disrupted IS*Ecp1* sequence in *K. pneumoniae* isolates. Lane1: AES64. Lane2: AES178. Lane3: AES261. Lane4: AES268. Lane5: AES273. Lane6: AES274. Lane7: AES280. Lane8: AES970. Lane9: AES973. Lane10: AES984. Lane11: AES1001. Lane12: Negative control. Lane13: Marker.

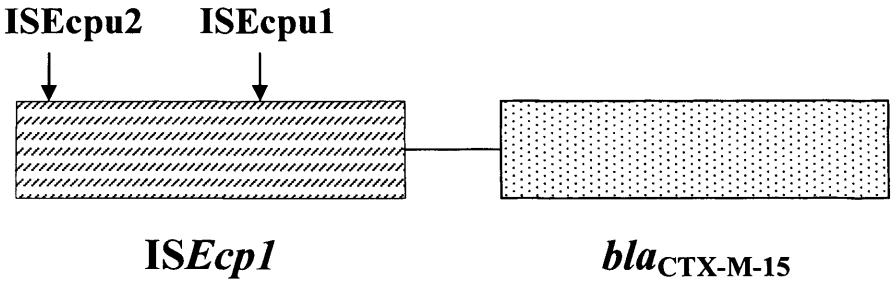

Figure 3.5 Diagram showing the genetic environment of $bla_{CTX\text{-}M\text{-}15}$ encoding gene and the insertion sequence IS*Ecp1* located upstream of the cefotaxime resistance gene. Arrows of ISEcpu1(Ho *et al.*, 2005) & ISEcpu2 (Leflon-Guibout *et al.*, 2004) indicates the target of each primer

3.2.2.3 Detection of TEM & SHV in *K. pneumoniae* isolates

Blotting of 80 *K. pneumoniae* isolates with TEM and SHV genes are presented in Figures 3.6A, 3.6B, 3.7A, 3.7B, 3.8A, 3.8B, 3.9A & 3.9B and the results are summarised in Table 3.3. These results showed that 52 (65%) isolates of *K. pneumoniae* were positive for bla_{SHV} genes and 27 (33.7%) were positive for bla_{TEM} genes. The occurrence of $bla_{CTX\text{-}M15}$, bla_{SHV} and bla_{TEM} genes together was detected in 12 isolates, whereas 16 isolates showed the both presence of bla_{SHV} and bla_{TEM}. The results also showed that bla_{SHV} genes were mostly detected in clinical settings (51.25%) compared to those *K. pneumoniae* isolates found in the hospital environment (10%). A low percentage of SHV genes were observed in environmental isolates collected outside the hospital. However, the incidence of bla_{TEM} among Libyan *K.*

pneumoniae in this study was 26.3% in clinical isolates and 7.5% in the hospital environment.

Table 3.3 The incidence of bla_{CTX-M} group1, Tn*402*, bla_{TEM} & bla_{SHV} encoding genes and mobile genetic elements *ISCR*2 in Libyan *K. pneumoniae* isolates

	Clinical isolates	Hospital environmental isolates	Environmental isolates	Total %
CTX-M group 1	**40 (n=80)**	**10**	**1**	**68.75%**
Tn*402*	**19 (n=80)**	**1**	**2**	**27.5%**
SHV	**41 (n=80)**	**8**	**3**	**65%**
TEM	**21 (n=80)**	**6**	**0**	**33.75%**
***ISCR*2**	**13 (n=80)**	**3**	**1**	**21.25%**

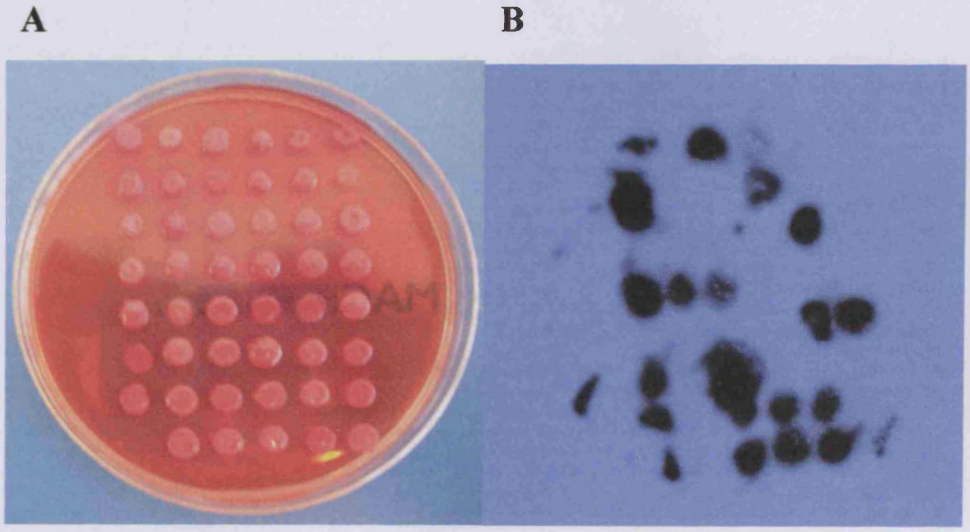

Figure 3.6 Blotting of *K. pneumoniae* isolates (1-47) and probing with bla_{TEM}. A. MacConkey Agar plate. B. Blotting and probing with radio-labelled bla_{TEM} of plate A.

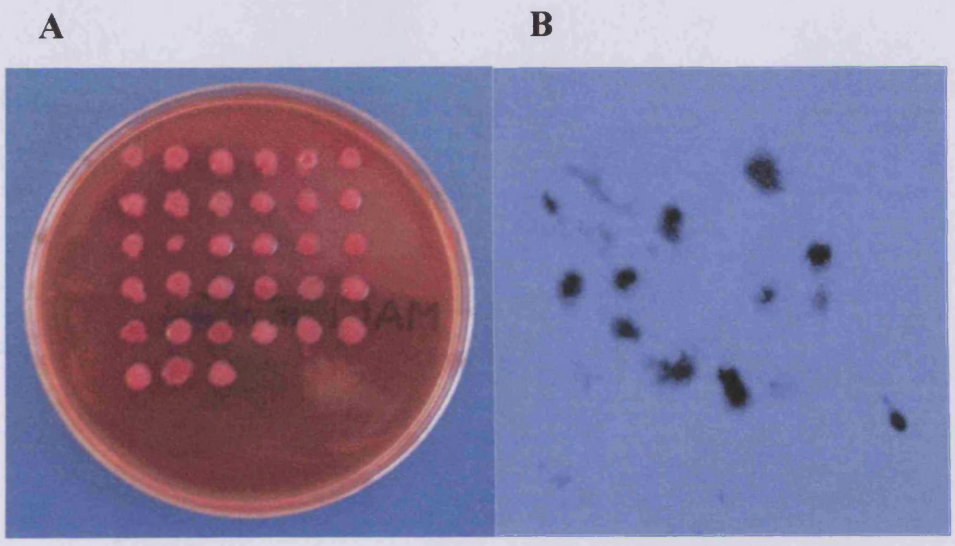

Figure 3.7 Blotting of *K. pneumoniae* isolates (48-80) and probing with bla_{TEM}. A. MacConkey Agar plate. B. Blotting and probing with radio-labelled bla_{TEM} of plate A.

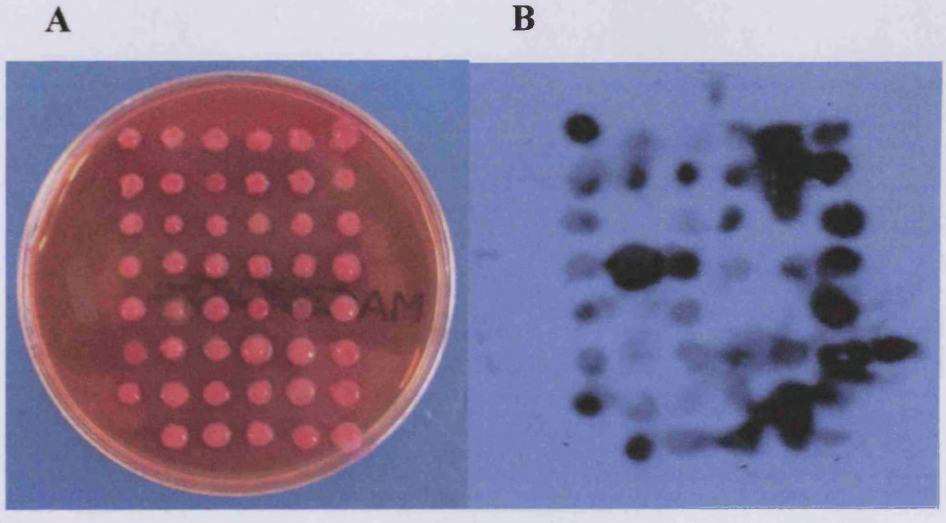

Figure 3.8 Blotting of *K. pneumoniae* isolates (1-47) and probing with bla_{SHV}. A. MacConkey Agar plate. B. Blotting and probing with radio-labelled bla_{SHV} of plate A.

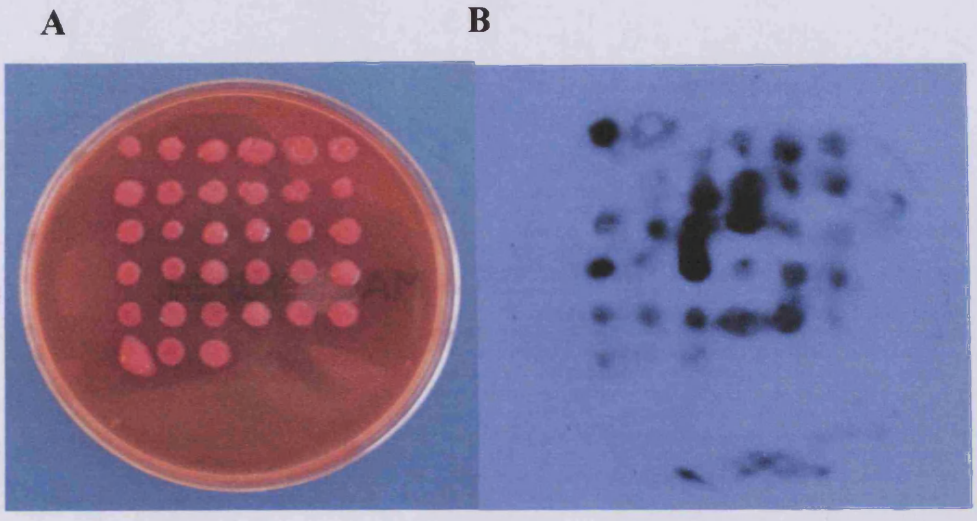

Figure 3.9 Blotting of *K. pneumoniae* isolates (48-80) and probing with bla_{SHV}. A. MacConkey Agar plate. B. Blotting and probing with radio-labelled bla_{SHV} gene of plate A.

3.2.2.4 CTX-M group 1 type ESBLs

Blotting and probing of 80 *K. pneumoniae* isolates with $bla_{CTX\text{-}M\text{-}15}$ template DNA, labelled with radioactive phosphorus ^{32}P, are summarised in Table 3.3 and illustrated in Figures 3.10A, 3.10B, 3.11A & 3.11B. 51/80 (63.8 %) were positive for $bla_{CTX\text{-}M}$ group 1 and 40 out of those 51 (78.4%) were isolated from blood, urine, pus, sputum, burn ward and sepsis samples collected from patients in different hospitals in Tripoli and Benghazi. The presence of $bla_{CTX\text{-}M}$ group 1 positive *K. pneumoniae* in the hospital environments was 10/51 (19.6%) and reflects the incidence and prevalence of $bla_{CTX\text{-}M}$ group 1 in Libyan hospitals. Thus, in total 50/51 $bla_{CTX\text{-}M}$ group 1 positive *K. pneumoniae* were from patients or the hospital environment and is very high compared to the spread of $bla_{CTX\text{-}M}$ group 1 genes in the community and

environment outside the hospitals. Only one isolate of *K. pneumoniae* collected from Benghazi streets was found carrying $bla_{CTX\text{-}M}$ group 1 genes.

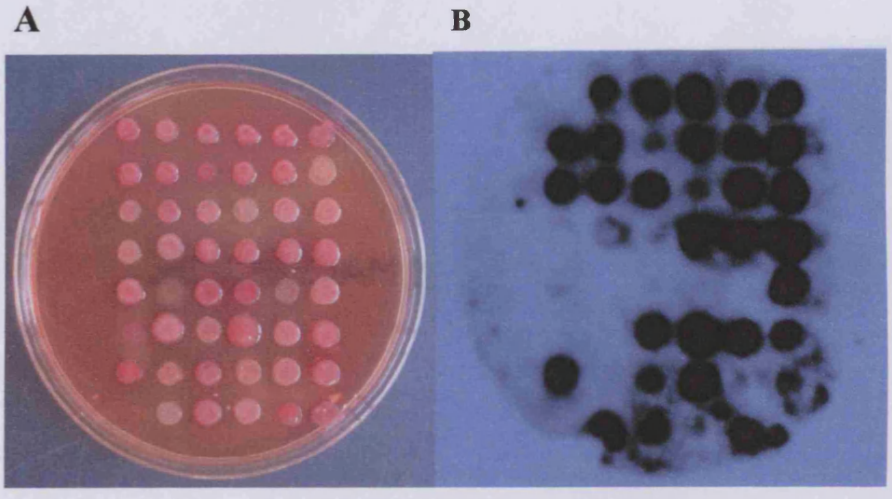

Figure 3.10 Blotting and probing of *K. pneumoniae* isolates (1-47) with $bla_{CTX\text{-}M\text{-}15}$. A: MacConkey Agar culture. B: Blotting and probing with radio-labelled $bla_{CTX\text{-}M\text{-}15}$ amplicon of plate A.

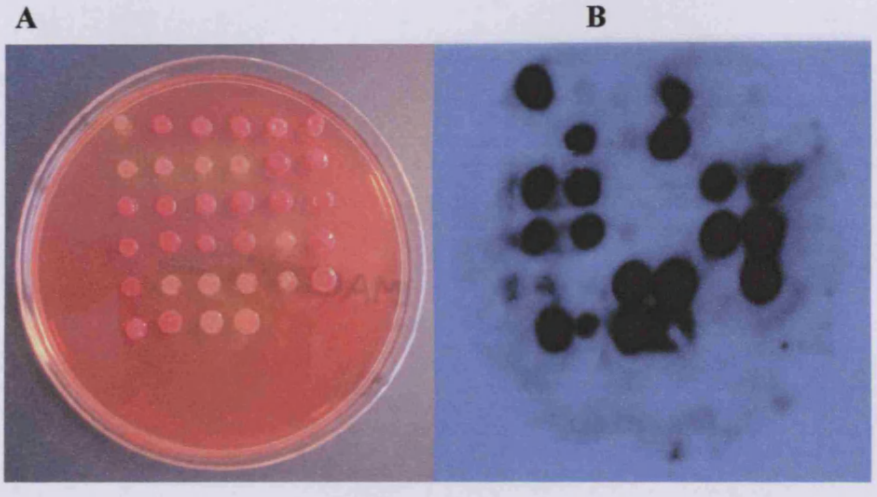

Figure 3.11 Blotting and probing of *K. pneumoniae* isolates (48-80) with $bla_{CTX\text{-}M\text{-}15}$. A: MacConkey Agar cultures. B: Blotting and probing with radio-labelled $bla_{CTX\text{-}M\text{-}15}$ amplicon of plate A.

3.2.2.5 Detection of *bla*$_{OXA-48}$ and IS*1999*

PCR experiments on *K. pneumoniae* failed to amplify *bla*$_{OXA-48}$ and IS*1999*.

3.2.3 Characterisation of plasmids carrying *bla*$_{CTX-M}$ group1/IS*Ecp1*

Figure 3.12 shows *S*1 endonuclease digestion followed by PFGE of genomic DNA separating chromosomal DNA from plasmids in 14/28 selected *K. pneumoniae* isolates (#AES8, AES48, AES135, AES140, AES141, AES216, AES274, AES275, AES279, AES280, AES281, AES506, AES722, AES808b, AES809E). An additional figure showing the same application with the other 14/28 *K. pneumoniae* isolates (AES809, AES817, AES203, AES836, AES939, AES961, AES942, AES188, AES994, AES960, AES970, AES975, AES977 & AES982) is presented in Appendix B.6. The selection criterion was based on prevalence of clinical samples but also included hospital environmental isolates and the single *K. pneumoniae* found in the streets. This experiment was undertaken to examine the incidence of plasmid mediated *bla*$_{CTX-M}$ group1 and IS*Ecp1* genes. Probing of the PFGE gel from Figure 3.12 with radio-labelled *bla*$_{CTX-M}$ group1/IS*Ecp1* is shown in Figure 3.13. Probing of the PFGE gel from figure B.6 is presented in Appendix B (Figure B.7). These results clearly demonstrated that *bla*$_{CTX-M}$ group1/IS*Ecp1* has been detected on plasmids in 14 isolates of *K. pneumoniae* on seven different plasmid sizes - 50, 75, 100, 150, 275, 300 and 425kb. Four isolates (AES8, AES135, AES140 & AES141) carry *bla*$_{CTX-M}$ group1/IS*Ecp1* on plasmids of 300kb, 3 isolates (AES506, AES970 & AES982) carry *bla*$_{CTX-M}$

group1/IS*Ecp1* on the same size of plasmids (175kb), whereas 3 isolates (AES274, AES280 & AES281) carrying bla_{CTX-M} group1/IS*Ecp1* on a 75kb plasmid. bla_{CTX-M} group1/IS*Ecp1* were found on a 100kb plasmid in *K. pneumoniae* isolate AES275 and in the hospital environmental isolate, AES722, on a plasmid of 50kb, and on a plasmid of 275kb in a clinical isolate, AES48, that was cultured from a blood sample. The *K. pneumoniae* isolate, AES817, found in on the Benghazi streets carry bla_{CTX-M} group1/IS*Ecp1* on a 425kb plasmid.

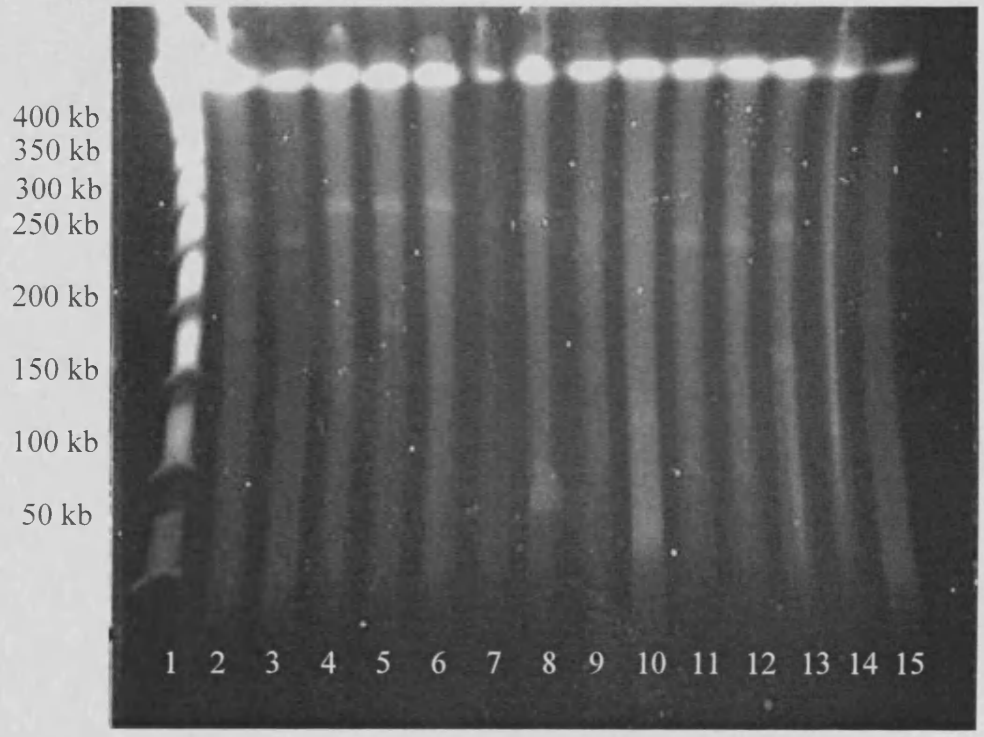

Figure 3.12 PFGE of *S*1 digests for *K. pneumoniae* AES isolates. Lane1: Marker. Lane2: AES8. Lane3: AES48. Lane4: AES135. Lane5: AES140. Lane6: AES141. Lane7: AES216. Lane8: AES274. Lane9: AES275. Lane10: AES279. Lane11: AES280. Lane12: AES281. Lane13: AES506. Lane14: AES722. Lane15: AES808B.

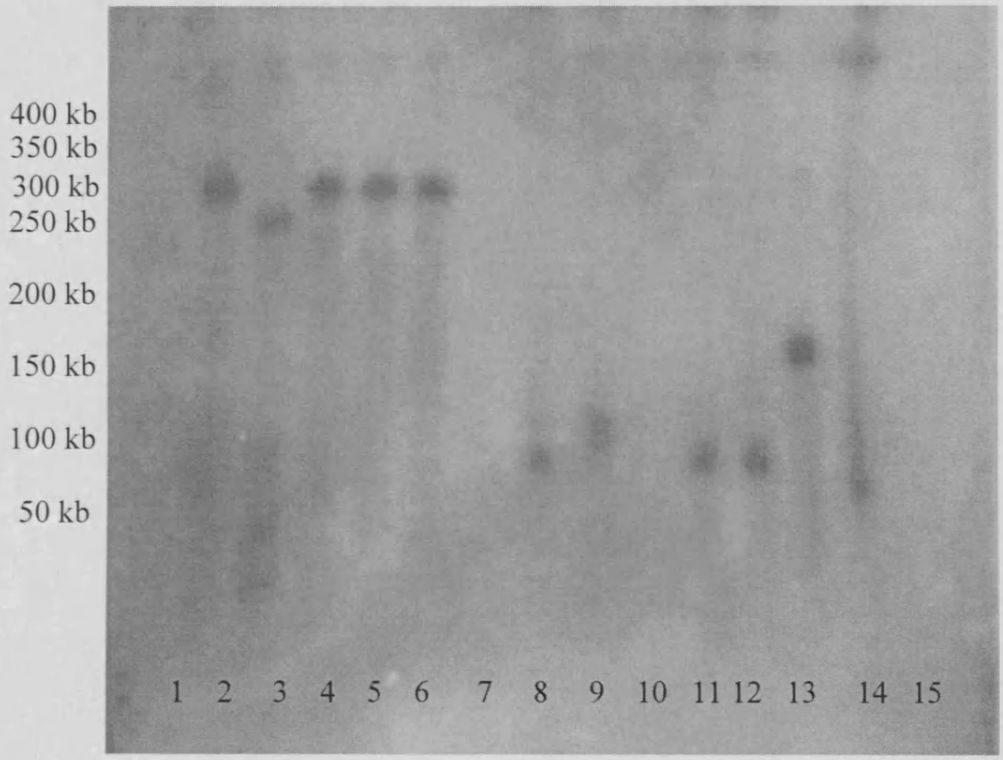

Figure 3.13 Autorad after probing with *bla*$_{CTX\text{-}M\text{-}15}$/IS*Ecp1* of blotted PFGE from Fig. 3.12. Lane1: Marker. Lane2: AES8. Lane3: AES48. Lane4: AES135. Lane5: AES140. Lane6: AES141. Lane7: AES216. Lane8: AES274. Lane9: AES275. Lane10: AES279. Lane11: AES280. Lane12: AES281. Lane13: AES506. Lane14: AES722. Lane15: AES808B.

3.2.4 Typing of *K. pneumoniae* by RAPD

Typing of 80 *K. pneumoniae* isolates by using RAPD technique are illustrated in Figure 3.14. The *K. pneumoniae* isolates can be divided into 6 clusters according to the Pearson correlation test that was performed using GelCompar software. Members of cluster 2 (n=32) displayed 85% similarity and 34/41 (82.9%) were only collected from patients in Tripoli and Benghazi and included sites such as blood, urine, sputum, pulmonary, CVL, pus samples, maternity hospital and burn and plastic surgery centre of Tripoli. Isolates of this cluster were collected as swabs from the hospital environments and also included the non-hospital environmental isolates. Cluster one included the isolates AES135 and AES140, AES172 and AES178 that appeared clonal when *Xba*I digestion was used (see section 3.2.5). Cluster 1 (n=26) also showed high similarity between members (90%), and 19/26 (73%) of the isolates were collected from blood, urine, sepsis and embilica samples, they were also cultured from maternity ward infections and burn ward infections. Isolates in cluster 2 were found in the hospital environments (bedsides, baby incubators, vacuum of suction machines, suction machine tubes and floor of toilets). One member of cluster 2 was isolated from the largest Benghazi Lake which is considered highly polluted. Members of cluster 4 (n=3) resembles cluster 3 as all members of this cluster were isolates collected from patients. Cluster 3 is composed of 6 members collected from a Tripoli maternity hospital and isolates AES982 and AES985 are clonal. Members of cluster 4 include two isolates (AES225 and AES261) that (by *Xba*I digestion of the

whole DNA) are clonal. Cluster 5 (n=6) includes isolates collected from patients and in addition to the high similarities (95%) between these members, they were also all positive for *bla*$_{CTX-M}$ group1.Cluster 6 (n=7) was different from the other clusters as members of that cluster share very low similarities (30%).

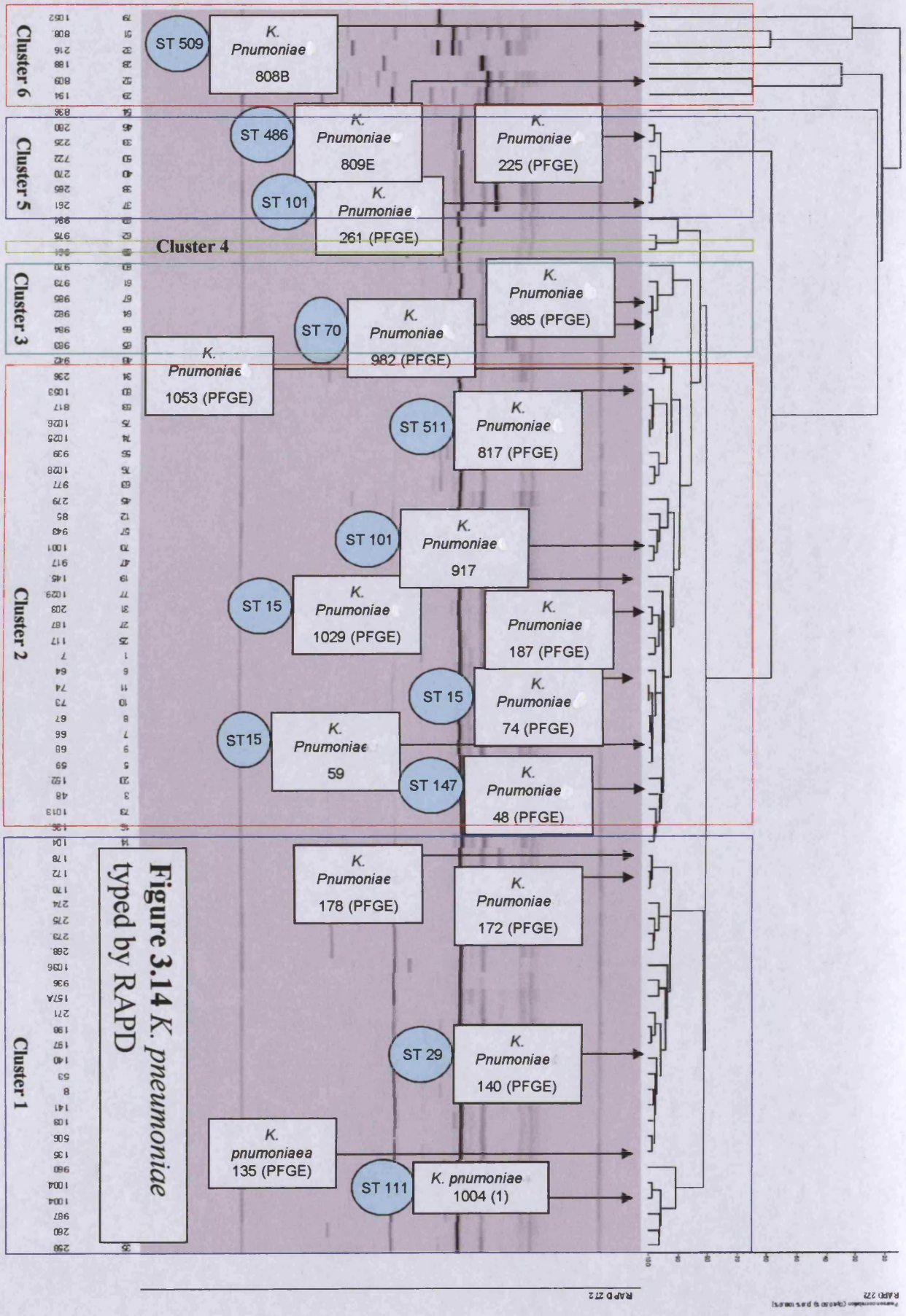

Figure 3.14 *K. pneumoniae* typed by RAPD

3.2.5 Molecular typing of *K. pneumoniae*

PFGE of *Xba*I digests of 28 *K. pneumoniae* isolates is shown in Figures 3.15 & 3.16 and the corresponding dendrograms shown in Figures 3.17 & 3.18. These results show that some isolates of *K. pneumoniae* are clonally related (>0.95) despite the different site of collection. Isolates AES135 and AES140 are clonal despite the fact that they were collected from two different hospitals; *K. pneumoniae* isolate AES135 was from a blood sample from a hospital in Benghazi city whereas *K. pneumoniae* isolate AES140 was from a urine sample from a patient in a hospital from a village near Benghazi. Isolates AES172 and AES178 are clonal. Isolate AES172 was from a baby incubator and isolate AES178 was collected from a vacuum suction machine. These two clonal isolates were found in the neonatal ICU in Benghazi Paediatric hospital. The results also show that *K. pneumoniae* isolates; AES273 and AES260 share a high-level of similarities (>0.90) and were collected from blood and umbilical samples, respectively. These two samples were collected from two different patients; however, the patients were admitted to the same hospital but not the same ward revealing the potential spread of the same clone within the hospital. *K. pneumoniae* isolates AES506 and AES1013 also share high similarities (>80%) despite being collected from two different hospitals in Tripoli; AES506 was collected from a suction machine tube in Tripoli Paediatric hospital, while AES1013 was from a patient admitted to Tripoli burn and plastic surgery centre of Tripoli. The other isolates of *K. pneumoniae*

that were examined by PFGE shared low level of similarities (<75%) showing that many strains of *K. pneumoniae* in Libya played a significant role in the spread of infection and antibiotic resistance genes.

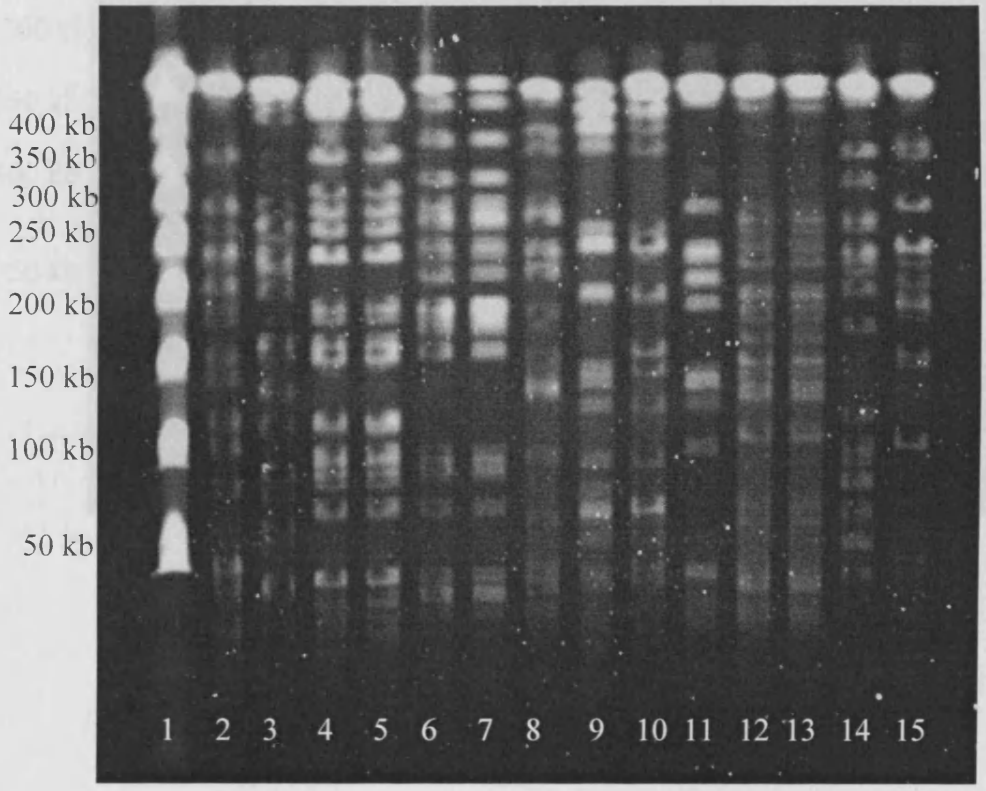

Figure 3.15 PFGE of *Xba*1 digests of *K. pneumoniae* genomic DNA. Lane1: Marker. Lane2: AES48. Lane3: AES74. Lane4: AES135. Lane5: AES140. Lane6: AES172. Lane7: AES178. Lane8: AES187. Lane9: AES225. Lane10: AES261. Lane11: AES817. Lane12: AES982. Lane13: AES985. Lane14: AES1029. Lane15: AES1053.

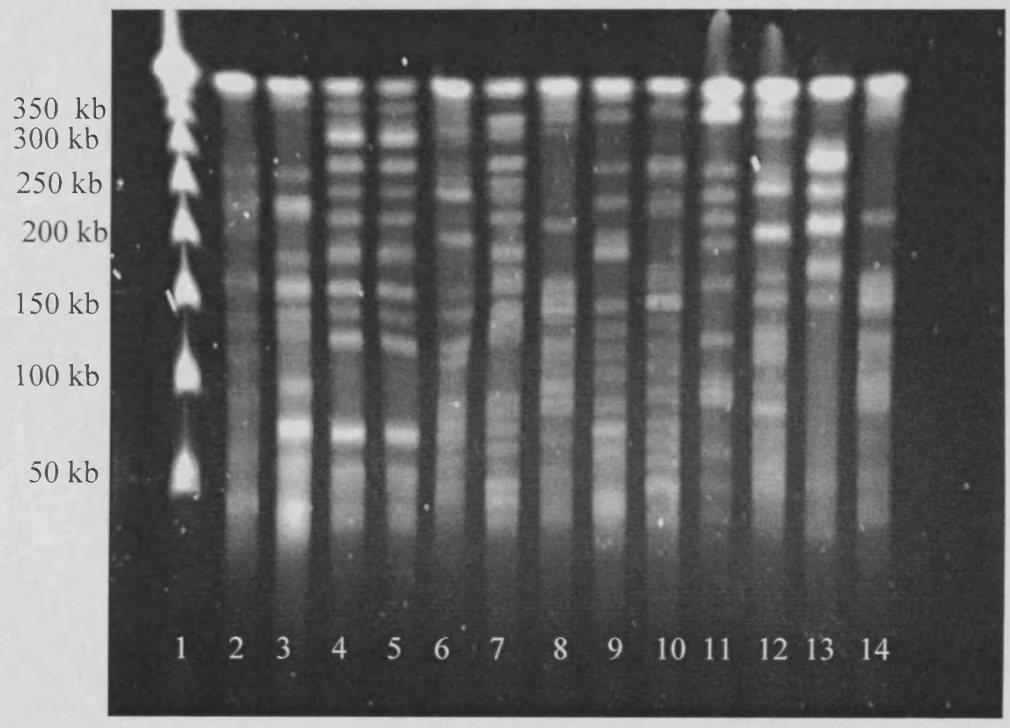

Figure 3.16 PFGE of *Xba*1 digests of *K. pneumoniae* genomic DNA. Lane1: Marker. Lane2: AES73. Lane3: AES203. Lane4: AES260. Lane5: AES273. Lane6: AES275. Lane7: AES506. Lane8: AES975. Lane9: AES977. Lane10: AES1004. Lane11: AES1013. Lane12: AES1026. Lane13: AES1028. Lane14: AES961.

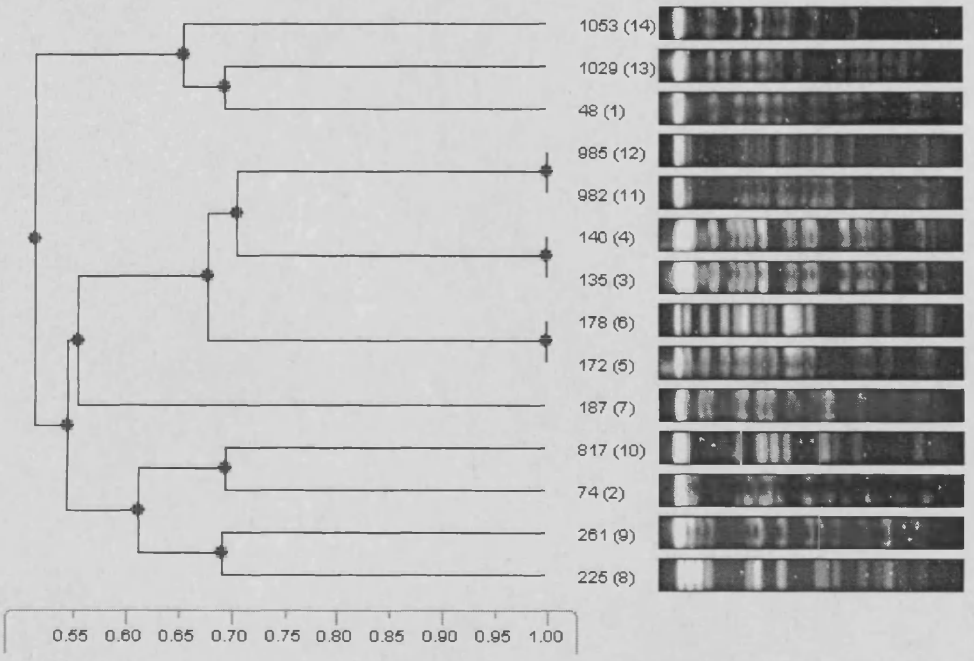

Figure 3.17 Dendrogram of PFGE gel showing *XbaI* digested DNA from *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES48. Lane3: AES74. Lane4: AES135. Lane5: AES140. Lane6: AES172. Lane7: AES178. Lane8: AES187. Lane9: AES225. Lane10: AES261. Lane11: AES817. Lane12: AES982. Lane13: AES985. Lane14: AES1029. Lane15: AES1053.

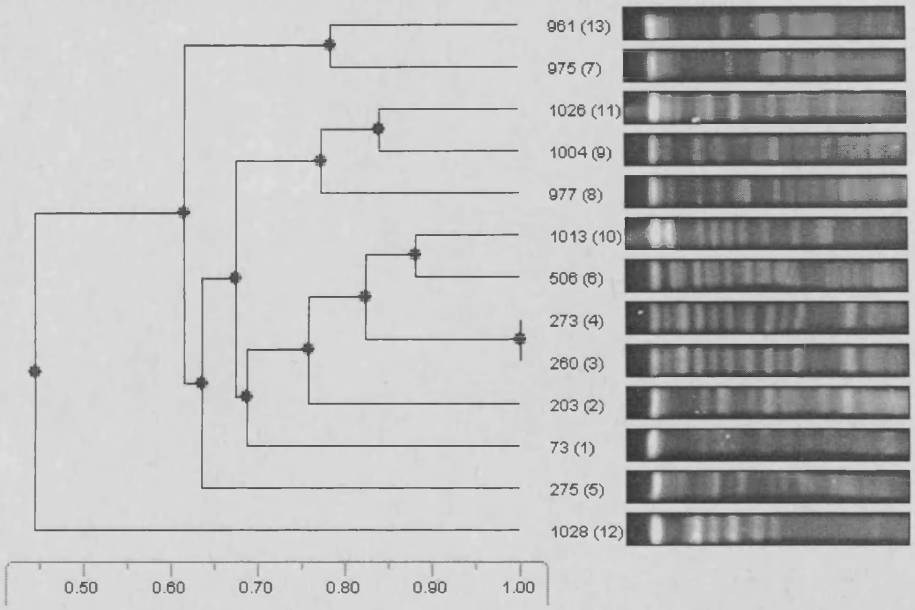

Figure 3.18 Dendrogram of PFGE gel showing *Xba*I digested DNA from *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES73. Lane3: AES203. Lane4: AES260. Lane5: AES273. Lane6: AES275. Lane7: AES506. Lane8: AES975. Lane9: AES977. Lane10: AES1004. Lane11: AES1013. Lane12: AES1026. Lane13: AES1028. Lane14: AES961.

3.2.6 Multi-locus sequence typing (MLST)

Representative isolates from RAPD-clusters were subjected to MLST. PCR experiments yielded PCR products of all housekeeping genes (see Appendix B). Generally, using RAPD fingerprinting typing method, similar RAPD-types gives similar sequence types and different RAPD-types give rise to different sequence types (STs). Sequencing of housekeeping genes of all 12 representative isolates of *K. pneumoniae* showed the occurrence of 9 sequence types among all isolates tested. The sequence types found were ST15, ST111, ST29, ST147, ST511, ST70, ST101, ST486 and ST509. ST15, ST111, ST29, ST147, ST70 and ST101 were among the clinical isolates whereas ST511, ST486 and ST509 were non-hospital environmental isolates from Benghazi. It is worthy of note that three isolates had ST15; AES59, AES74 and AES1029. AES Isolates 59 and 74 were collected from mechanical ventilators from an ICU ward of the 7th of October hospital in Benghazi, whereas AES1029 was a clinical isolate from a patient admitted to a Burn ward in Alkhadra hospital in Tripoli. These STs were from clusters which shared more than 90% similarities and part of one large cluster which included 17 members. One exception was observed, with ST101 being observed in two unrelated RAPD-clusters sharing less than 60% similarities. The isolates that had ST101 were; AES261 which was a clinical isolate recovered from a blood sample from Al-Jamhoryia hospital in Benghazi and AES isolate 917 which was from a curtain on an ICU ward in Al-Jala hospital in Benghazi. Nevertheless, both isolates

were detected positive for *bla*$_{CTX-M}$ group 1. The most frequently observed sequence type was ST15, which has earlier been described in *bla*$_{CTX-M-15}$-producing *K. pneumoniae* (Damjanova *et al.*, 2008). Also, ST15 is a single locus variant (SLV) of ST14, which has been described in *bla*$_{CTX-M}$, *bla*$_{KPC}$ and *bla*$_{NDM-1}$ producing *K. pneumoniae* (Hrabak *et* al., 2009; Oteo *et al.*, 2009; Kitchel *et al.*, 2009; Samuelsen *et al.*, 2011) Two other sequence types, ST147 and ST101 have also been linked to the dissemination of *bla*$_{CTX-M}$ in previous reports. (Hrabak *et al.*, 2009; Damjanova *et al.*, 2008). ST29 was a clinical isolate from blood and was also positive for *bla*$_{CTX-M-15}$. This ST has earlier been described in extended-spectrum cephalosporin-resistant isolates, but has not been frequently reported recently (Diancourt *et al.*, 2005). ST70 was a clinical isolate from Tripoli maternity hospital and positive for *bla*$_{CTX-M}$ group 1, while ST111 was a clinical isolate recovered from a patient in burn and plastic surgery centre of Tripoli, and was also positive for *bla*$_{CTX\_M}$ group 1. ST70 and ST111 have not been associated with dissemination of CTX-M-producing *K. pneumoniae* in previous reports, and are not closely related to any of the main epidemic clones. The novel sequence type ST511(http://www.pasteur.fr/cgi-bin/genopole/ PF8/mlstdbnet.pl ?file=klebs_ profiles.xml&page=profileinfo&st=511) is an environmental isolate cultured from a swab collected from one of the Benghazi streets. This isolate carries a plasmid mediated *bla*$_{CTX-M-15}$ and is a double-locus variant of ST35 and ST36 which have both recently been described in CTX-M-producers (Oteo *et al.*, 2009). Two environmental isolates were new sequence types, ST486

(http://www.pasteur.fr/cgibin/genopole/PF8/mlstdbnet.pl?file=klebs_profiles.xml&page=profileinfo&st=486) and ST509 (http://www.pasteur.fr/cgi-bin/genopole/PF8/mlstdbnepl?file=klebs_profiles.xml&page=profileinfo&st=509). These isolates were also cultured from a swab collected from two different roads in Benghazi.

3.2.7 Detection of chromosomally and plasmid mediated bla_{CTX-M} group1

Probing of PFGE of *Xba*I digests of *K. pneumoniae* (figures 3.15 & 3.16) with radio-labelled $bla_{CTX-M-15}$ template DNA is shown in (Figures 3.19 & 3.20). *K. pneumoniae* AES48, AES135 and AES140 possesses four copies of bla_{CTX-M} group 1 in different locations including the various plasmids. *K. pneumoniae* AES74, AES172, AES178, AES225, AES1029, AES1053, AES260, AES273, AES275 and AES1026 possess two copies of bla_{CTX-M} group1 genes. Only one copy of bla_{CTX-M} group 1 gene was detected in *K. pneumoniae* AES261, AES817, AES982, AES985, AES73, AES506 & AES1004. The incidence of more than one copy of bla_{CTX-M} group 1 gene in some isolates of *K. pneumoniae* might raise the question of how can bla_{CTX-M} group 1 genes move within the genome of *K. pneumoniae*, Such movement could be facilitated by the active presence of IS*Ecp1* which can mobilise bla_{CTX-M} group 1. Digestion with Xba*I* does not discriminate plasmid from chromosome and therefore the bands seen in Figures 3.19 & 3.20 can only refer to the number of copies of bla_{CTX-M} group 1 and not their genetic location.

3.2.8 Transconjugation Experiments

Transconjugation experiments on 51 *K. pneumoniae* positive for bla_{CTX-M} group1 showed that successful transfer of resistance occurred in 27/51 (52.9%). PCR analysis on transconjugants confirmed the movement of bla_{CTX-M} group 1 and it is promoter sequence IS*Ecp1* from parents of *K. pneumoniae* to transconjugants (*E. coli*) (Figures 3.21 & 3.22). Sequencing of these alleles showed the occurrence of bla_{CTX-M} group1 and IS*Ecp1* in the new generation of transconjugants and further confirmed the movement capability of bla_{CTX-M} group1/IS*Ecp1* from parents to recipients, indicating the role of conjugative plasmids in transfer. Some transconjugation experiments failed to transfer bla_{CTX-M} group1 assuming the non-conjugative plasmid location of bla_{CTX-M} group1 and/or IS*Ecp1* or the occurrence of one copy of chromosomal located bla_{CTX-M} group1.

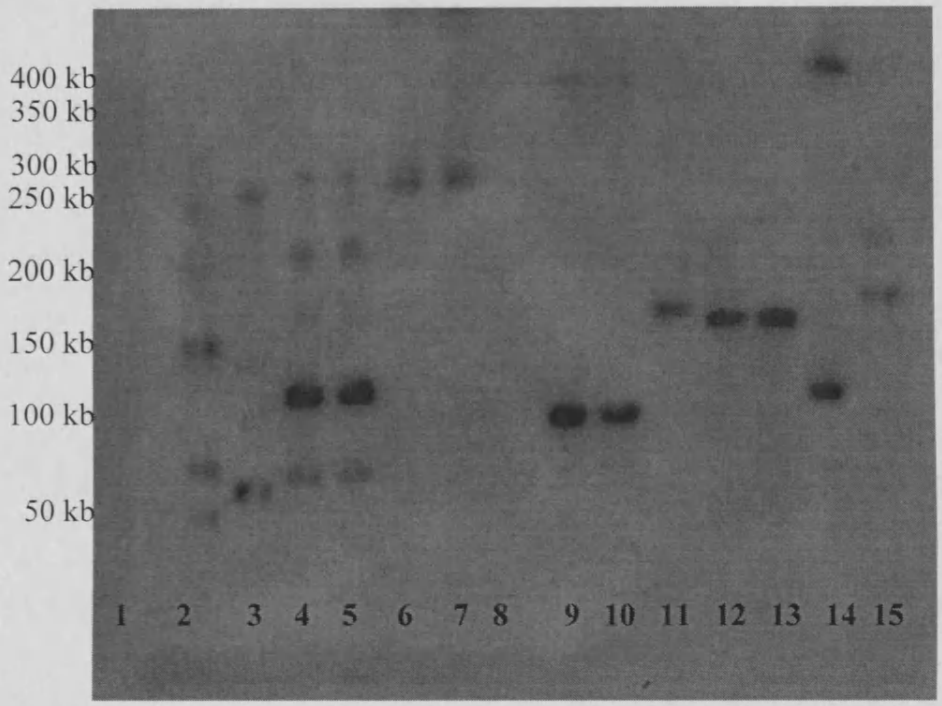

Figure 3.19 Autorad after probing with $bla_{\text{CTX-M-15}}$ of blotted PFGE from Fig. 3.15. Lane1: Marker. Lane2: AES48. Lane3: AES74. Lane4: AES135. Lane5: AES140. Lane6: AES172. Lane7: AES178. Lane8: AES2187. Lane9: AES225. Lane10: AES261. Lane11: AES817. Lane12: AES982. Lane13: AES985. Lane14: AES1029. Lane15: AES1053.

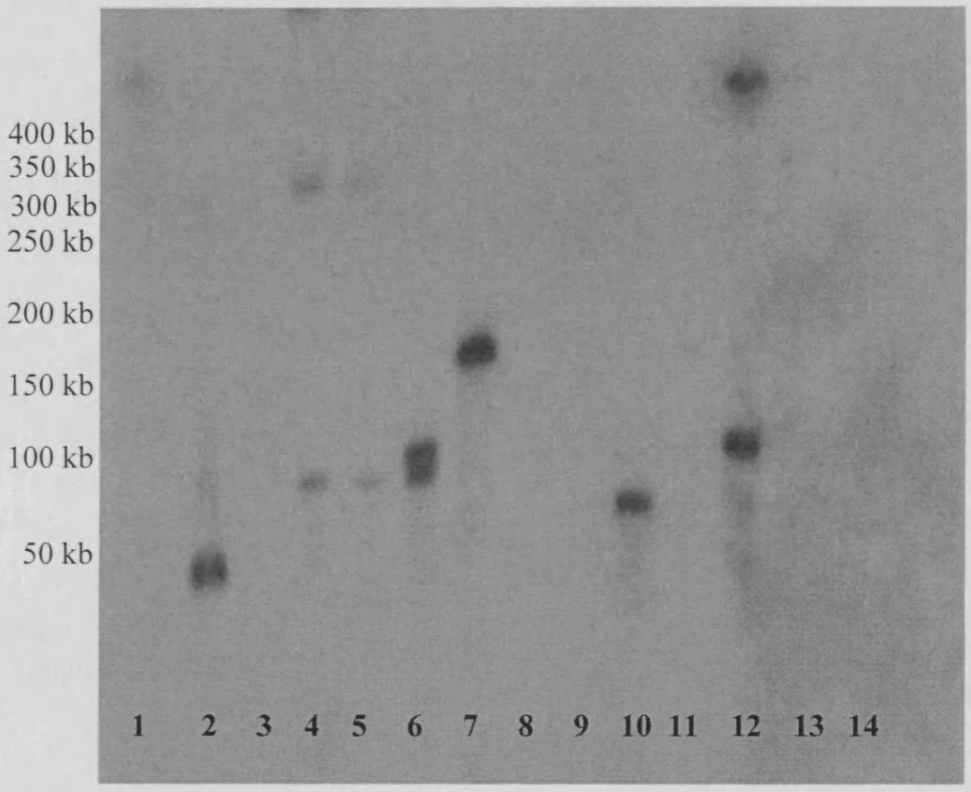

Figure 3.20 Autorad after probing with $bla_{CTX\text{-}M\text{-}15}$ of blotted PFGE from Fig. 3.16. Lane1: Marker. Lane2: AES73. Lane3: AES203. Lane4: AES260. Lane5: AES273. Lane6: AES275. Lane7: AES506. Lane8: AES975. Lane9: AES977. Lane10: AES1004. Lane11: AES1013. Lane12: AES1026. Lane13: AES1028. Lane14: AES961.

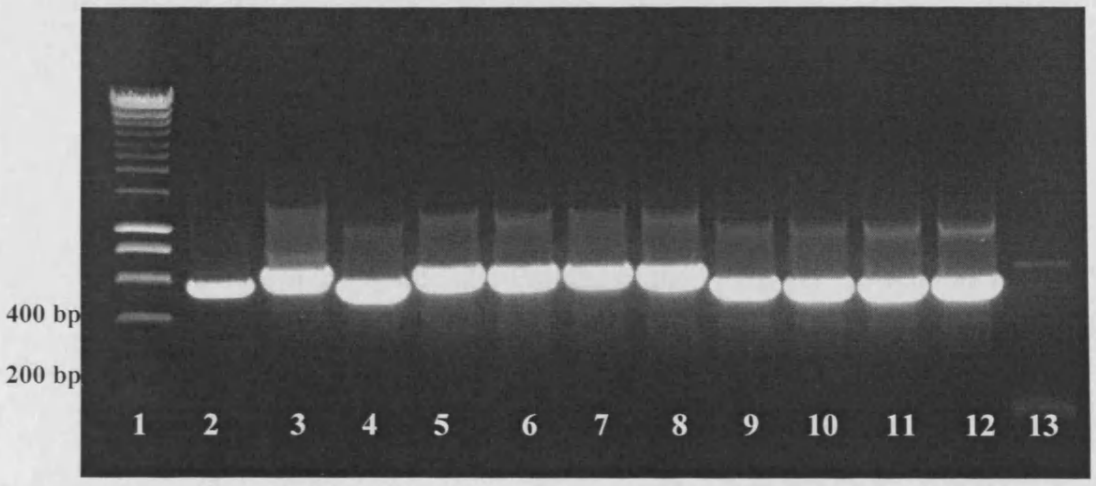

Figure 3.21 Detection of *bla*$_{CTX-M}$ group1/IS*Ecp1* in GFP *E. coli* transconjugants of *K. pneumoniae* AES isolates. Lane1: Marker. Lane2: AES74T. Lane3: AES178T. Lane4: AES261T. Lane5: AES268T. Lane6: AES273T. Lane7: AES274T. Lane8: AES280T. Lane9: AES970T. Lane10: AES975T. Lane11: AES984T. Lane12: AES1001T. Lane13: negative control.

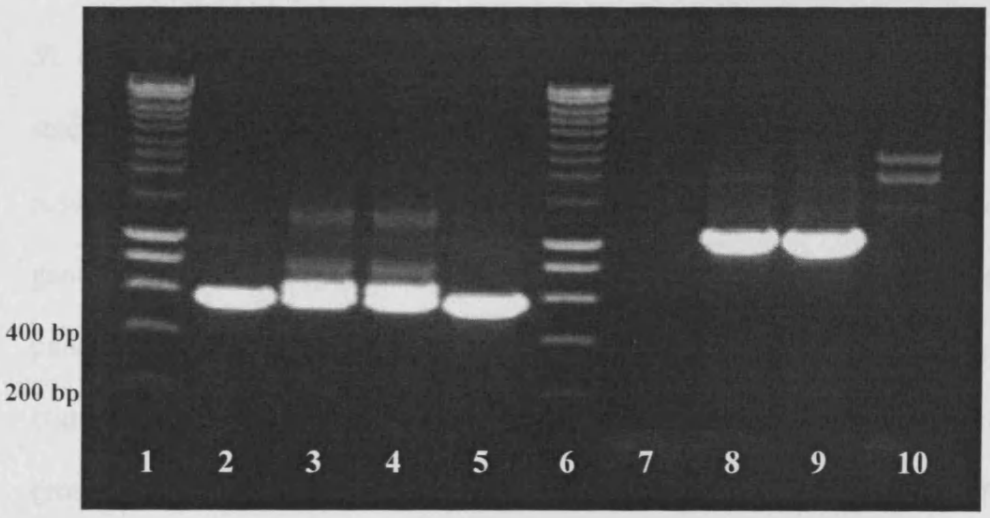

Figure 3.22 Detection of the occurrence of an intact (2-5) and disrupted (7-10) copies of IS*Ecp1* in GFP *E. coli* transconjugants of *K. pneumoniae* AES isolates. Lane1: Marker. Lane2: AES74T. Lane3: AES172T. Lane4: AES178T. Lane5: AES268. Lane6: Marker. Lane7: AES74T. Lane8: AES172T. Lane9: AES178T. Lane10: AES268T.

3.2.9 Detection of plasmid mediated *bla*$_{CTX-M}$ group1 in parents and transconjugants

PFGE separation of *S*1 endonuclease digestion of genomic DNA from a subset of 6 parents of *K. pneumoniae* and their 6 recipients of *E. coli* are shown in Figure 3.23. The result of the probed PFGE gel with a custom made *bla*$_{CTX-M-15}$ probe is shown in Figure 3.24. Probing of the PFGE gel showed that *bla*$_{CTX-M}$ group1 have successfully transferred to *E. coli* as the recipient. The results clearly confirm the plasmid location and also demonstrated that the plasmid carrying *bla*$_{CTX-M}$ group1 has moved, more or less, unaltered. Intriguingly, the data from Fig. 3.24 also shows that some of the copies of *bla*$_{CTX-M}$ group1 are chromosomal a phenomenon not well cited in the literature.

3.2.10 Detection of the movement of IS*Ecp1* from parents to transconjugants

*S*1 endonuclease digestion and separation of genomic DNA by PFGE of a selection of parents and transconjugants are illustrated in Figure 3.25. The results of probing of the PFGE gel with radio-labelled *bla*$_{CTX-M-15}$1/IS*Ecp1* genes are shown in Figure 3.26. These results show the same size plasmids in parents and transconjugants. The results clearly demonstrate the capability of clinical and non-clinical isolates of Libyan *K. pneumoniae* to acquire *bla*$_{CTX-M}$ group1/IS*Ecp1* and to confer such a resistance mechanism to recipients of *E. coli*. *bla*$_{CTX-M}$ group1/IS*Ecp1* have been detected on a plasmid of 300kb in isolates AES74, AES135, AES140 and AES141 and their respective recipients. *bla*$_{CTX-M}$ group1/IS*Ecp1* was also detected on a 100kb plasmid in

AES172, AES172T, AES178 and AES178T. AES48 demonstrates the incidence of 5 copies of *bla*$_{CTX-M}$ group1/IS*Ecp1* on plasmids of different sizes - 50, 100, 200, 250 and 300kb.

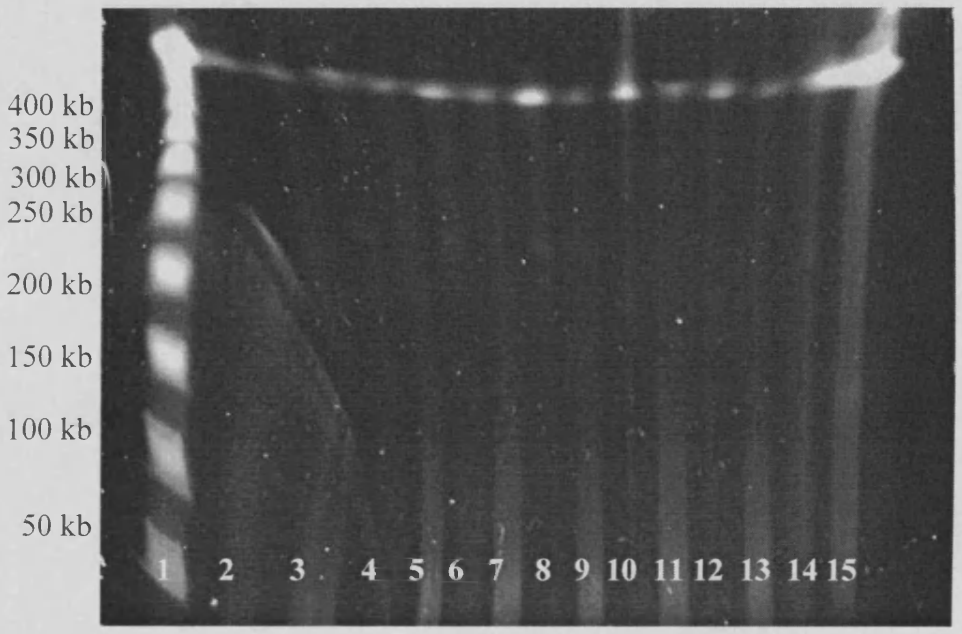

Figure 3.23 PFGE of *S*1 digests of *K. pneumoniae* and GFP transconjugants. Lane1: Marker. Lane2: AES74. Lane3: AES74T. Lane4: AES135. Lane5: AES135T. Lane6: AES140. Lane7: AES140T. Lane8: AES2141. Lane9: AES141T. Lane10: AES172. Lane11: AES172T. Lane12: AES178. Lane13: AES178T. Lane14: AES48 (positive control). Lane15: 5738 (positive control).

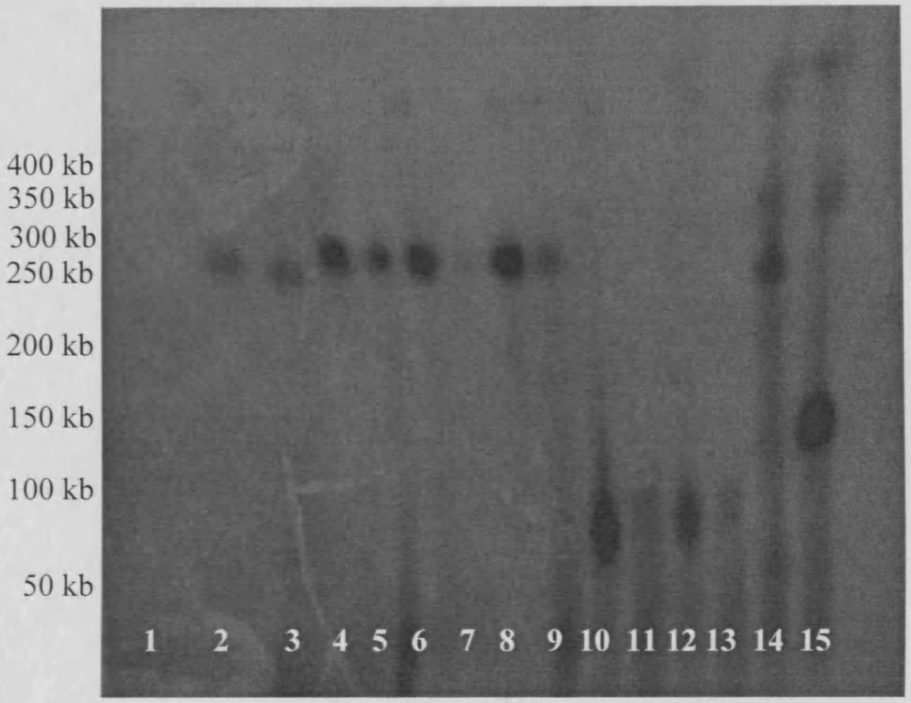

Figure 3.24 Autorad after probing with *bla*$_{\text{CTX-M-15}}$ of blotted PFGE from Fig. 3.23. Lane1: Marker. Lane2: AES74. Lane3: AES74T. Lane4: AES135. Lane5: AES135T. Lane6: AES140. Lane7: AES140T. Lane8: AES2141. Lane9: AES141T. Lane10: AES172. Lane11: AES172T. Lane12: AES178. Lane13: AES178T. Lane14: AES48 (positive control). Lane15: 5738 (positive control).

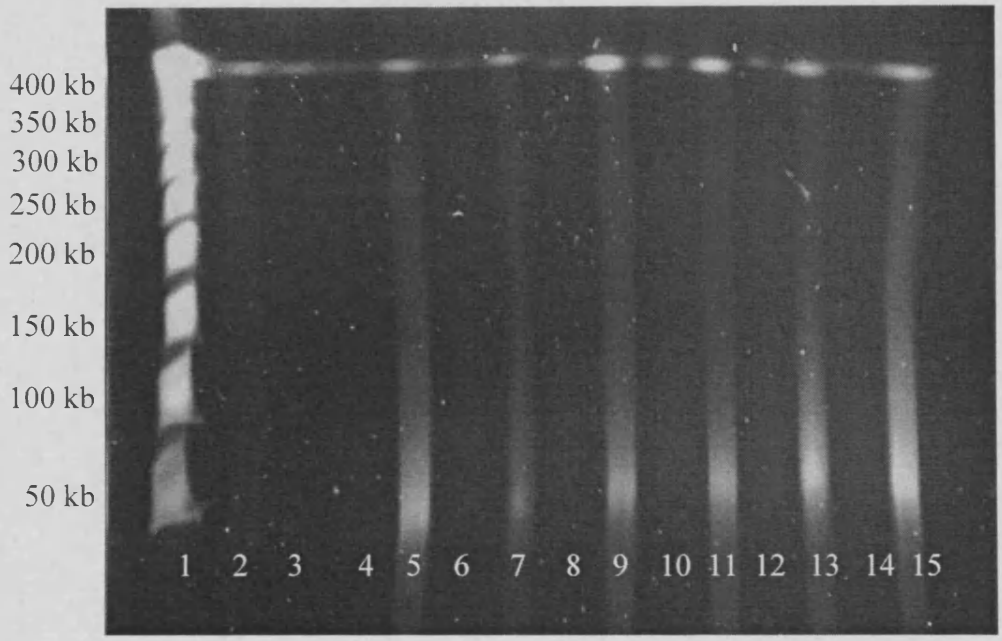

Figure 3.25 PFGE of *S*1 digests of *K. pneumoniae* and GFP transconjugants. Lane1: Marker. Lane2: AES48 (positive control). Lane3: AES1052 (Negative control). Lane4: AES74. Lane5: AES74T. Lane6: AES135. Lane7: AES135T. Lane8: AES2140. Lane9: AES140T. Lane10: AES141. Lane11: AES141T. Lane12: AES172. Lane13: AES172T. Lane14: AES178. Lane15: AES178T.

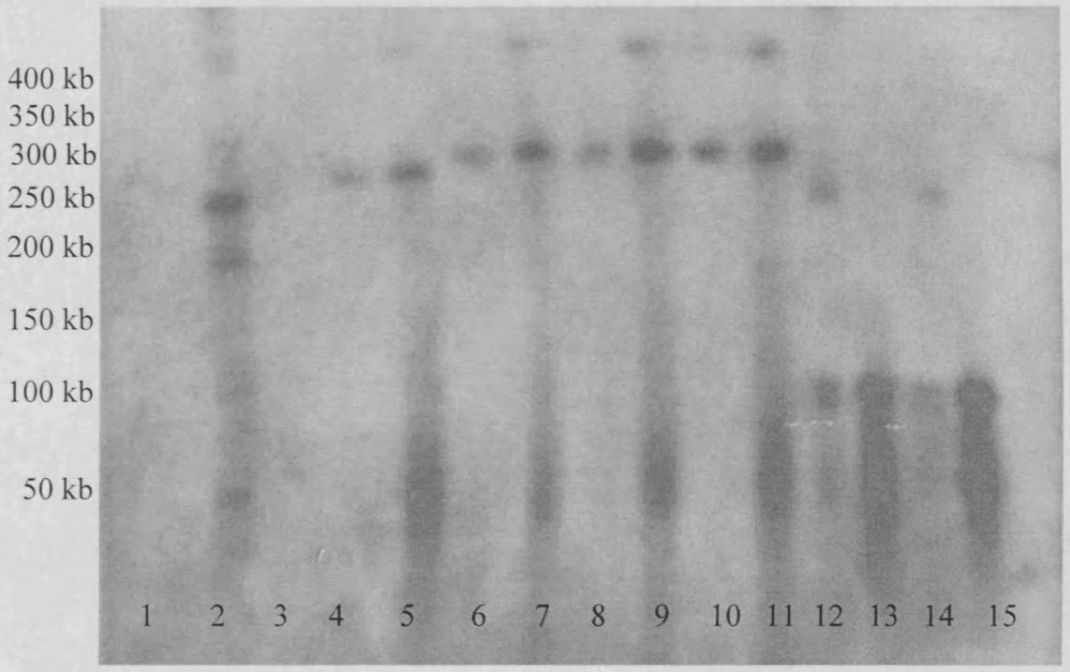

Figure 3.26 Autorad after probing with *bla*$_{\text{CTX-M-15}}$/IS*Ecp*1 of blotted gel from Fig. 3.25. Lane1: Marker. Lane2: AES48 (positive control). Lane3: AES1052 (Negative control). Lane4: AES74. Lane5: AES74T. Lane6: AES135. Lane7: AES135T. Lane8: AES2140. Lane9: AES140T. Lane10: AES141. Lane11: AES141T. Lane12: AES172. Lane13: AES172T. Lane14: AES178. Lane15: AES178T. (T: transconjugate of respective parent)

3.2.11 Plasmid Typing

PCR reactions failed to produce any *inc/rep* PCR products of the *K. pneumoniae* plasmids; nevertheless, *inc/rep* PCR products were detected on the positive control reference plasmids. These results suggest that these plasmids are non-typeable. They also suggest that the plasmids responsible for carrying $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M}}$ group1 are significantly different from those already characterised by Carattoli *et al.*, 2005 which to date is considered the most recent and applicable system for detecting conjugative plasmids.

3.2.12 Detection of mobile genetic elements

3.2.12.1 Class 1 integrons

The results of PCR reactions yielded PCR products of different sizes and copies in 20/ 22 (90.90 %) randomly selected isolates (Figure 3.27). Isolates AES8, AES85, AES179, AES198, AES271A, AES280, AES135 and AES140 produce a 1kb class 1 integrons whereas isolates AES48, AES59, AES66 and AES74 were positive for a 1.5kb integron. Two copies of class 1 integrons were found in *K. pneumoniae* isolate AES48. Sequencing of these alleles showed 4 different genetic contexts (B.8 - B.17). The differences between these integrons depend on the number and type of gene cassettes embedded in these integrons. *K. pneumoniae* isolates AES179, AES198, AES271, AES280, AES8, AES135 and AES140 share the same class 1 integron genetic context. This integron is composed of an integrase gene and dihydrofolate reductase genes that confers resistance to trimethoprim (*dfrA*30), and resistance to

sulphamethoxazole (*qac*EΔ/*sul*1) (Figure 3.28B). Integron of AES135 was submitted to the gene bank and assigned accession numbers; HE613850.1, HE613852.1, HE613851.1 and HE613853.1. Class 1 integrons detected in *K. pneumoniae* isolates AES59, AES66 and AES74 were found sharing the same genetic context; an integrase gene and a dihydrofolate reductase type VII (*dfrA*17) which confer resistance to trimethoprim and an aminoglycoside-3'-adenyltransferase resistance gene (*aad*A5) flanked with the conserved region *qac*EΔ/*sul*1 (Figure 3.28C). The occurrence of 3 destinct integrons was identified in *K. pneumoniae* isolates AES48 (Figure 3.28D) and AES85 (Figure 3.28A). AES48 had a class 1 integron composed of an integrase gene, *dfrA*12 and *aad*A2 which is known to confer resistance to streptomycin and spectinomycin, and *qac*EΔ/*sul*1. Only one gene cassette, *dfrA*7, was found embedded in the integron of AES85 (Figure 3.28).

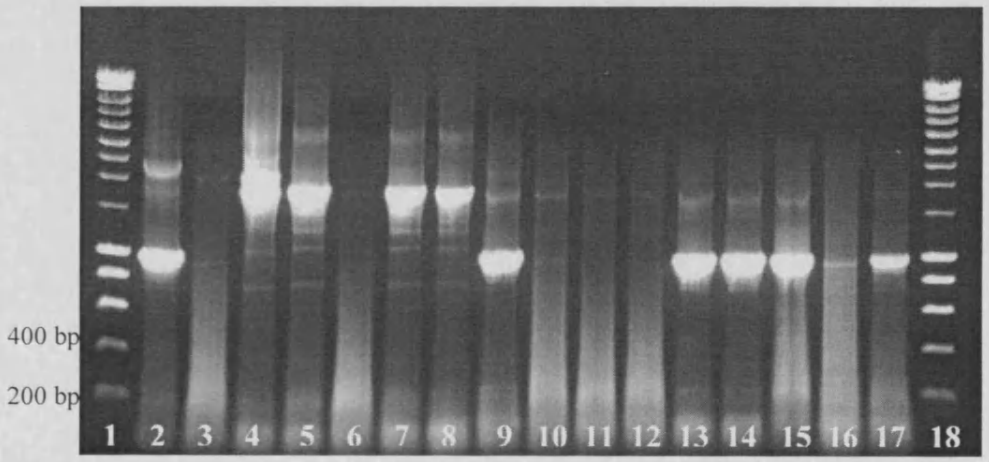

Figure 3.27 Amplification of the classical class 1 integrons. Lane1: Marker. Lane2: AES8. Lane3: AES25. Lane4: AES48. Lane5: AES59. Lane6: AES64. Lane7: AES66. Lane8: AES74. Lane9: AES85. Lane10: AES170. Lane11: AES172. Lane12: AES178. Lane13: AES179. Lane14: AS198. Lane15: AES271. Lane16: AES275. Lane17: AES280. Lane18: Marker

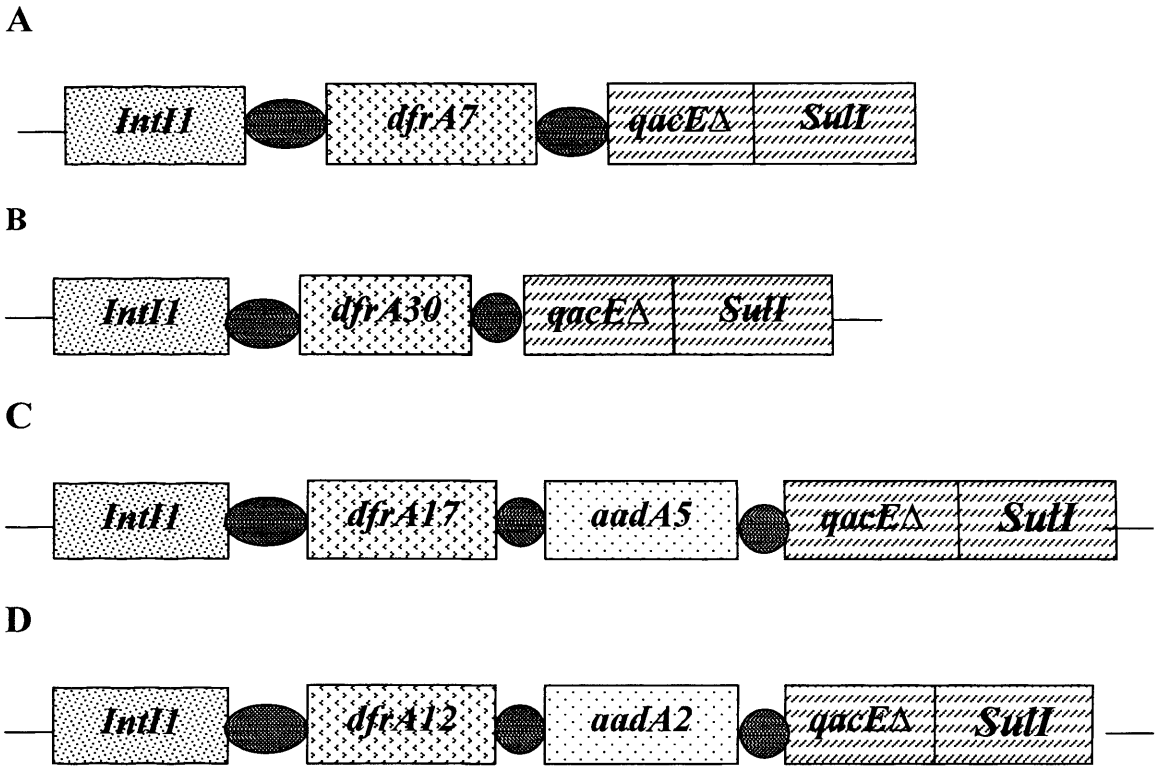

Figure 3.28 Genetic context of 6 class 1 integrons found in *K. pneumoniae* isolates. A: Class 1 integron from AES85. B: Class 1 integron from isolates; AES198, AES179, AES271, AES280, AES8, AES135 & AES140. C: Class 1 integron from isolates; AES74, AES66 & AES59. D: Class 1 integron from isolate; AES48.

3.2.12.2 Identification of Tn*402* transposons

Amplification of *tni*C gene (a marker for Tn*402*) was detected in 14/20 (70 %) isolates randomly examined (Figure 3.29). Sequencing of PCR products of 3 isolates of *K. pneumoniae* showed the occurrence of two different types of Tn*402* type transposons in three isolates of *K. pneumoniae* - AES135, AES197 and AES258. Isolate AES135 was also positive for the presence of class 1 integron (Figure 3.30). The transposon was found composed of an integrase gene, the trimethoprim resistance gene (*dfrA*30), *qac*E, and *tni*C (Figure 3.30). These results show the presence of Tn*402* transposons in both clinical and non-clinical isolates of *K. pneumoniae* (listed in Table 3.3). As judged by colony blotting only 22/80 (27.5 %) were positive for *tni*C type transposons, and 16/22 (72.7 %) of these transposon positive isolates were also positive for $bla_{\text{CTX-M}}$ group1. In spite of the low occurrence of Tn*402* compared with class 1 integrons, PCR data indicates that some isolates possess more than one copy of *tni*C.

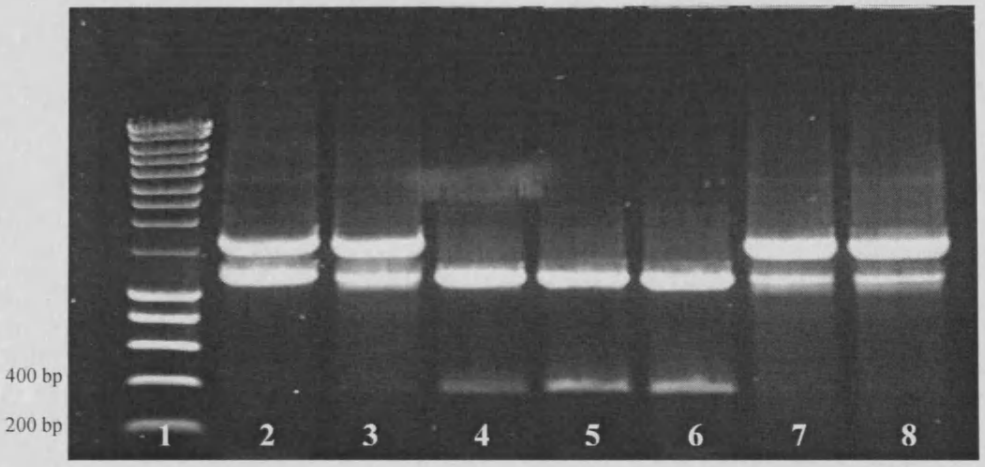

Figure 3.29 Detection of Tn*402* type transposons among *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES135. Lane3: AES140. Lane4: AES141. Lane5: AES157A. Lane6: AES170. Lane7: AES198. Lane8: AES258.

A

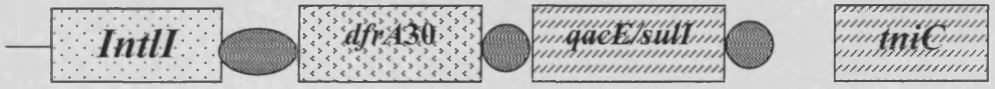

Figure 3.30 Genetic contexts of two Tn*402* type transposons found in *K. pneumoniae* isolates. A: transposon from AES135

3.2.12.3 Transposase Encoding Genes

3.2.12.3.1 PFGE of *S*1 genomic digests and probing with *tni*C

PFGE of *S*1 digest of genomic DNA and separation of plasmid according to size are shown in Figures 3.31 & 3.33. Probing of the PFGE gel with radio-labelled *tni*C gene is shown in Figures 3.32 & 3.34. Tn*402* was detected 13 isolates of *K. pneumoniae*, 5/13 (38.5 %) carry two copies of the transposon on 6 different sizes of plasmids of approximately 10, 15, 50, 60, 75 and 100 kb. Two isolates, AES135 and AES140, carry the transposon on a plasmid of 250kb and another isolate, AES157A, carries the transposon on a plasmid of 175kb. Isolates AES179, AES198 and AES258 have a Tn*402* transposon carried on a plasmid of 200kb. These transposons can act as gene capturing systems and contribute in the dissemination of antibiotic resistance genes by carrying genes responsible for conferring antibiotic resistance as part of class 1 integrons (Sajjad *et al.*, 2011).

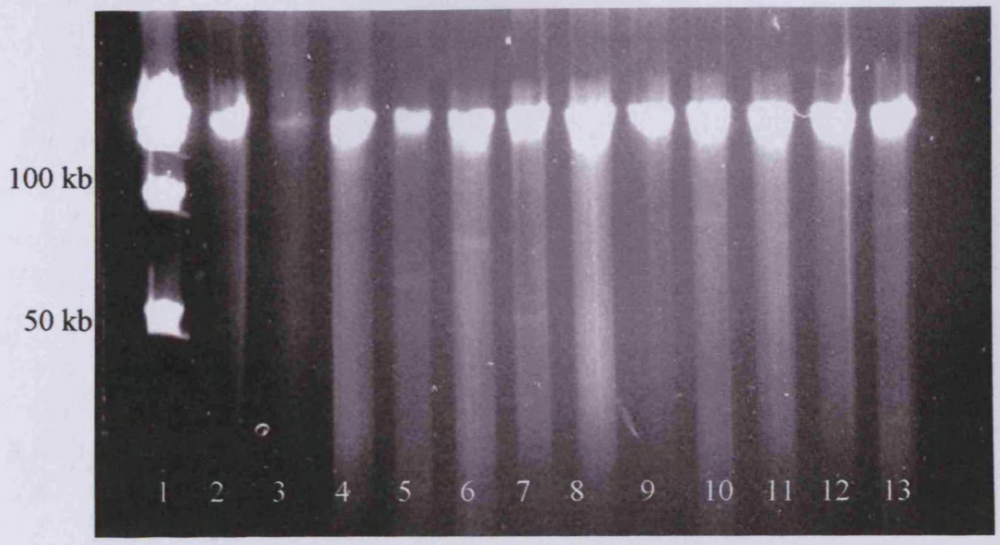

Figure 3.31 PFGE of *S*1 digests of *K. pneumoniae*. Lane1: Marker. Lane2: AES7. Lane3: AES8. Lane4: AES10. Lane5: AES25. Lane6: AES27. Lane7: AES48. Lane8: AES53. Lane9: AES59. Lane10: AES64. Lane11: AES66. Lane12: AES67. Lane13: AES68.

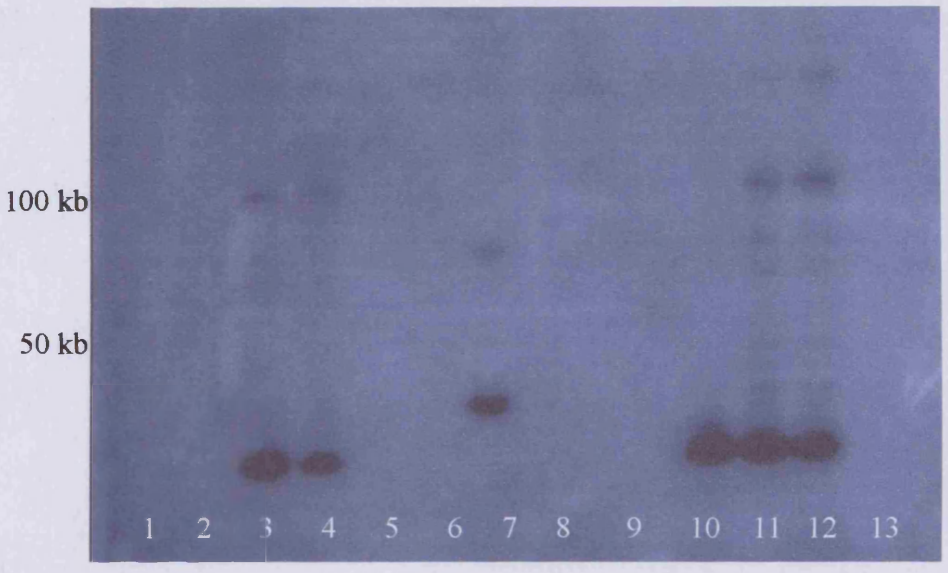

Figure 3.32 Autorad after probing with tniC of blotted gel from Fig. 3.31. Lane1: Marker. Lane2: AES7. Lane3: AES8. Lane4: AES10. Lane5: AES25. Lane6: AES27. Lane7: AES48. Lane8: AES53. Lane9: AES59. Lane10: AES64. Lane11: AES66. Lane12: AES67. Lane13: AES68.

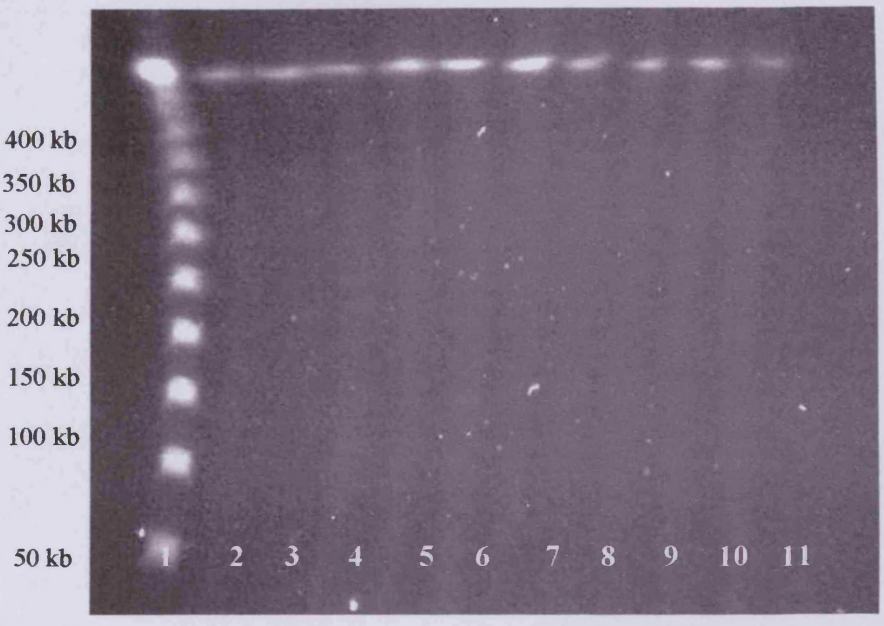

Figure 3.33 PFGE of *S*1 digests of *K. pneumoniae* genomic DNA. Lane1: Marker. Lane2: AES7. Lane3: AES135. Lane4: AES140. Lane5: AES152. Lane6: AES157. Lane7: AES172. Lane8: AES178. Lane9: AES179. Lane10: AES198. Lane11:

Figure 3.34 Autorad after probing with *tni*C of blotted PFGE gel from Fig. 3.33. Lane1: Marker. Lane2: AES7. Lane3: AES135. Lane4: AES140. Lane5: AES152. Lane6: AES157. Lane7: AES172. Lane8: AES2178. Lane9: AES179. Lane10: AES198. Lane11: AES258.

3.2.12.4 Detection of IS*CR* Elements

Probing of *K. pneumoniae* isolates with IS*CR2* genes is presented in (Table 3.3) and (Figures 3.35A, 3.35B, 3.36A & 3.36B). 17 isolates were positive for IS*CR2*. Of these, 12/17 (70.5 %) were also positive for $bla_{CTX\text{-}M}$ group1. 5/17 (29.41 %) were also positive for Tn*402*. 13/17 (76.47 %) *K. pneumoniae* isolates possessing IS*CR2* were from patients whereas, 3/17 (17.6 %) were isolates found in the hospital environment. Only one *K. pneumoniae* strain collected from the broader Benghazi environment was positive for $bla_{CTX\text{-}M}$ group1, IS*Ecp1* and IS*CR2* and also showed successful transconjugation.

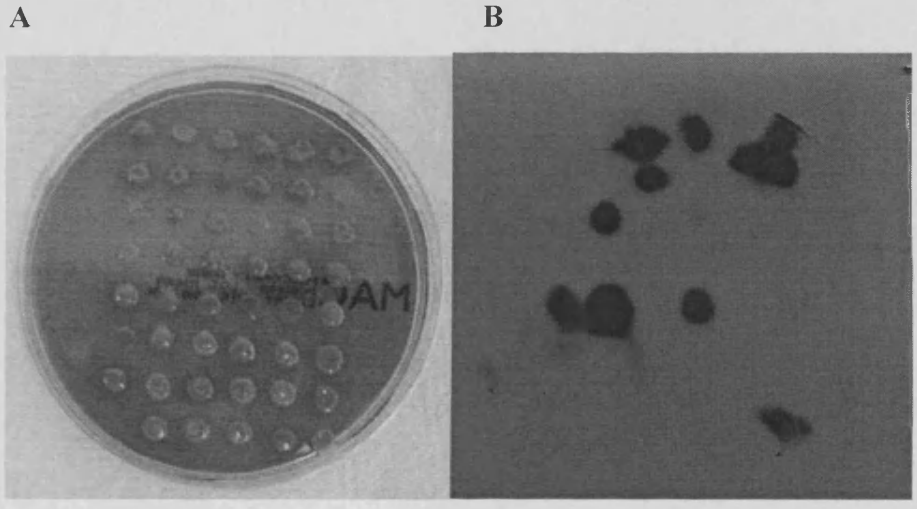

Figure 3.35 Probing of blotted *K. pneumoniae* isolates (1-47) with the IS*CR2* gene. A: *K. pneumoniae* isolates on MacConkey Agar. B: Autorad of blotting after probing with IS*CR2* gene.

A B

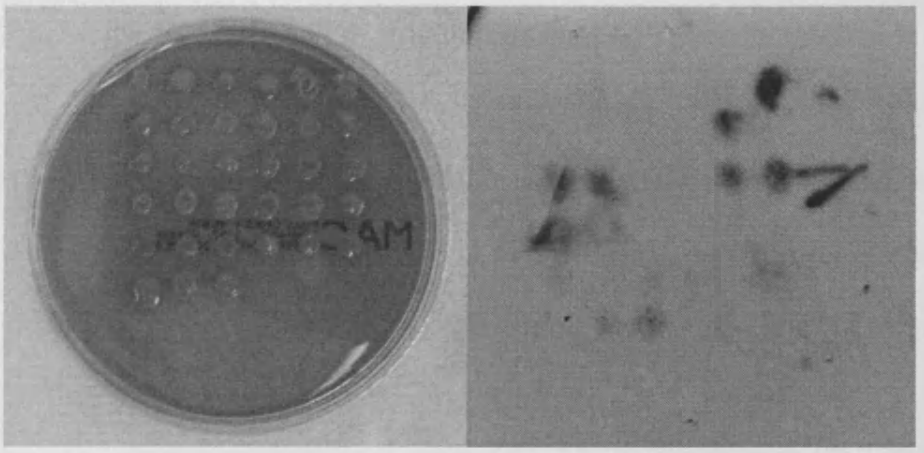

Figure 3.36 Probing of blotted *K. pneumoniae* isolates (48-80) with IS*CR2* gene. A: *K. pneumoniae* isolates on MacConkey Agar. B: Autorad of northern blotting after probing blotted plate A with IS*CR2* gene

3.3 Discussion

Due to the fact that there is little information on the current rate of infection and the spread of resistant strains of Gram-negative bacteria in Libya, this study was conducted to examine the resistance mechanisms (in some cases, in detail, a randomly selected subset) of *K. pneumoniae* isolates from Tripoli and Benghazi collected from the clinical settings and the environment outside the hospitals.

In addition to the fact that this study represents the first molecular analysis of antibiotic resistance on Gram-negative bacteria from Libya and in particular *K. pneumonia,* it has also major findings. The incidence of $bla_{\text{CTX-M}}$ group1 type ESBLs and the prevalence of chromosomally and plasmid mediated $bla_{\text{CTX-M-15}}$/IS*Ecp1* and $bla_{\text{CTX-M}}$ group1 among *K. pneumoniae* isolates are a key factor for their resistance. In addition these isolates are able to confer and express third generation cephalosporin resistance to sensitive *E. coli* via conjugative plasmids. Furthermore, the occurrence of clonally related isolates, in addition to the occurrence of new sequence types among *K. pneumoniae* is a major finding of this study. The involvement of class 1 integrons and Tn*402* type transposons as genetic mobile elements in some of these isolates aid spread of antibiotic resistance genes in Libyan hospitals.

The prevalence rate of CTX-M group1 genes in this study is markedly higher than the percentage reported in Algeria, Europe, USA and Canada (Messai *et*

al., 2008). Figures 3.10, 3.11, 3.13, 3.19, 3.20, 3.24 and 3.26 clearly demonstrate the incidence of $bla_{\text{CTX-M}}$ group1/IS*Ecp1* in clinical, non-clinical and environmental isolates of *K. pneumoniae*. It shows the dissemination of MDR *K. pneumoniae* in the clinical settings more in than the environment outside the hospitals. These findings are in accordance with the results of Mamlouk *et al.*, 2006, who reported a high incidence of $bla_{\text{CTX-M}}$ group1 in clinical specimens in Tunisia. The increasingly spread of $bla_{\text{CTX-M-}}$ group1 in Libya is likely due to the high consumption of the antibiotics cefotaxime and ceftazidime in the last ten years to treat infections in Libya. It might also be due to the lack of hygiene in hospital, such as hand hygiene, sterilisation, infection control and lack of surveillance programmes that is desperately lacking in Libya.

SHV and TEM type ESBL genes have also been found prevalent as high percentages among clinical isolates (78.8% and 77.7% respectively). bla_{SHV} and bla_{TEM} were also detected in the non-clinical isolates collected from floors, curtains, hospital equipment, and surfaces of baby incubators. SHV type ESBLs were also detected in environmental strains collected from streets. These findings illustrate the increased level of resistance in clinical isolates of *K. pneumoniae* and also highlight the depressing reality that this resistance is widespread across Libya and that resistance, in this instance, has got very little to do with the consumption of antibiotics.

bla$_{CTX-M}$ group1 was detected carried on 7 different plasmid sizes in 14 isolates of *K. pneumoniae*. Ten isolates were clinical samples, 3 were from the hospital environment and one isolate was from Benghazi streets. Overall, the occurrence of plasmid mediated *bla*$_{CTX-M}$ group1IS*Ecp1* seems to be higher in the clinical settings. *bla*$_{CTX-M}$ group1/IS*Ecp1* was found in 6 different hospitals in Tripoli and Benghazi on different plasmid sizes and locations. *bla*$_{CTX-M}$ group1/IS*Ecp1* was found on a plasmid of 300kb in 4 clinical samples, two of which were clonally related from two different hospitals in Benghazi; Jamhoryia and Kwaifia hospitals. These findings show the incidence of *bla*$_{CTX-M}$ group1/IS*Ecp1* in clonally and non-clonally related isolates of *K. pneumoniae*. This group of ESBLs was also located on a plasmid of 75kb in 3 clinical isolates of *K. pneumoniae* (AES274, AES280 and AES281) and on plasmids of 100kb and 275kb in the clinical isolates AES275 and AES48 respectively that were collected from Jamhoryia hospital. Although the same gene, with its promoter sequence was found on a plasmid of 150kb in *K. pneumoniae* clinical isolates AES982 and AES970 collected from Al-Jala Maternity hospital in Tripoli, they were found on the same plasmid size in the hospital environmental isolate AES506 swabbed from Al-Jala Paediatric hospital in Tripoli. It is worth mentioning that Al-Jala Paediatric hospital is located in Tripoli city centre and next to Al-Jala Maternity hospital. This might explain the occurrence of the *bla*$_{CTX-M}$ group1 in clinical isolates and the hospital environmental isolates despite being clonally unrelated according to RAPD test.

A plasmid mediated *bla*$_{CTX-M}$ group1/IS*Ecp1* was detected on a large plasmid, sized 425kb in a *K. pneumoniae* isolated from one of Benghazi streets. A possible explanation for the relatively low frequency of plasmid or chromosomally mediated *bla*$_{CTX-M}$ group1 in the streets could be because of the effect of the environment conditions outside the clinical settings. The results of this work are in agreement somewhat with the findings of (Lavollay, *et al.*, 2006) in terms of the wide range of the occurrence of *bla*$_{CTX-M-15}$ on plasmids of different sizes. These results are also consistent with the findings of the spread of plasmid mediated ESBLs that have been reported in *K. pneumoniae* strains in Europe and USA (Gori *et al.*, 1996) and Tunisia (Elhani *et al.*, 2010). The work described in this section conflicts somewhat with the findings of Gonullu *et al.*, 2008 who found that most *bla*$_{CTX-M-15}$/IS*Ecp1* were found in most cases located on a plasmid of the same size and type – in this cane IncN. The results of this section are also dissimilar to the work of (Messai *et al.*, 2008) who reported the prevalence of CTX-M genes on plasmids of approximately 77kb and 85kb.

Several important clones, which were recently found associated with spread of *bla*$_{CTX-M}$ and/or carbapenemases were described in this study. Hence, the study provides further support to the assumption that epidemic international clones are responsible for a substantial part of dissemination of *bla*$_{CTX-M}$ among *K. pneumoniae*. Transconjugation and detection of the movement of plasmid

mediated $bla_{CTX\text{-}M}$ group1 has been detected in *K. pneumoniae* ST15, ST29, ST101 and the new environmental allele ST511. Plasmid mediated $bla_{CTX\text{-}M}$ group1 has also been detected in *K. pneumoniae* ST147, ST111 and ST70. The spread of $bla_{CTX\text{-}M\text{-}15}$ producing *K. pneumoniae* has moreover been discovered in ST101 and ST147 in Tunisia (Elhani *et al*, 2010) and in this case Libyan patients might serve as a reservoir of such sequence types of *K. pneumoniae* as Libyans travel frequently to Tunisia in particular for medical purposes, cosmetic surgery and other medical necessities.

Determination of class 1 integrons and transposons by different methods showed the incidence of 5 genetic context forms of class 1 integrons in 12 isolates of *K. pneumoniae*. Some isolates shared the same genetic context while others had a different integron each. Isolate AES85 was found in a CVL sample and was positive for $bla_{CTX\text{-}M}$ group1/IS*Ecp1* and a globally distributed class 1 integron. The integron found in this isolate contained *Int*I, *dfrA*7 and *qac*EΔ/sulI. Several authors report the incidence of this integron in a number of clinical isolates - *S. typhi* serotype Typhi from Jordan, Nepal, Senegal, Uganda and South Africa (Al-Sanouri *et al.*, 2008; Tamang *et al.*, 2007; Sow *et al.*, 2007; Krauland *et al.*, 2009). An identical integron was, in addition found in clinical isolates of *E. coli* and *K. pneumoniae* from Sweden (Brolund *et al.*, 2010), in UTI clinical isolate of *E. coli* from Korea (Yu *et al.*, 2004) and in *Shigella flexneri* from Spanish patients who had visited Kenya.

Collectively, the high prevalence and abundance of *bla*$_{CTX-M}$ group1 and the occurence of *bla*$_{CTX-M-15}$ on its own and in association with IS*Ecp1*, *bla*$_{SHV}$, *bla*$_{TEM,}$ classical class 1 integron alone or embedded in transposon Tn*402*, indicate that the epidemiology of *K. pneumoniae* in Libyan hospitals is complex and probably reflects the existence of a longstanding infection control problems in each hospital. The data also indicates that resistance outside the hospital environment and in the community is also an issue.

Chapter Four

Characterisation of antibiotic resistance in *E. coli* isolates from Tripoli & Benghazi, Libya

4.1 Introduction

E. coli is a major cause of infections in humans and plays a significant role in nosocomial and CAIs particularly UTIs and bacteraemia among all ages of humans (Oteo *et al.*, 2010a; Rogers *et al.*, 2011; Oteo *et al.*, 2010b). ESBLs emerged in late 1980s causing healthcare associated infections that were now resistant to extended-spectrum β-lactamases and have spread worldwide (Apisarnthanarak *et al.*, 2008; Kiratisin *et al.*, 2008). In particular, plasmids mediated ESBLs. It is a probably the result of the extensive use of β-lactam antibiotics (Goyal *et al.*, 2009) and the selective pressure of these antibiotics which has caused the spread of plasmids from one pathogen isolate to another.

$bla_{\text{CTX-M}}$ genes encode for CTX-M enzymes, these genes are often plasmid encoded and known as narrow-host range plasmids. CTX-M type enzymes are among the most prevalent ESBLs in Europe, North America, Asia, Latin America and Africa (Gonullu *et al.*, 2008). It has been reported in Tunisia, Algeria, Lebanon and Egypt (Khalaf *et al.*, 2009). This type of ESBLs can be moved from bacteria to bacteria by means of transferable plasmids via conjugation. These enzymes, particularly the early ones that were discovered, preferably hydrolyse cefotaxime more than ceftazidime (Dhanji *et al.*, 2011). $bla_{\text{CTX-M-15}}$ ESBLs is the most frequently reported hydrolysing enzyme in the UK, Italy, Turkey, Spain, Australia, Kuwait, Lebanon, Algeria and Tunisia (Randall *et al.*, 2011; Cerquetti *et al.*, 2010; Gonullu *et al.*, 2008; Diaz *et al.*, 2010; Ensor *et al.*, 2009; Sidjabat *et al.*, 2010; Abbassi *et al.*, 2008; Mohamed-

Al-Agmy *et al.*, 2006). The outbreak of clonally related strains of *E. coli* has been reported in association with the incidence of ESBLs (Abbassi *et al.*, 2008; Woodford *et al.*, 2004). In view of the increasing world wide emergence of ESBLs and because there is no detailed information on the occurrence of ESBLs in Libya this study was carried out to study the prevalence of antibiotic resistance in 39 clinical and non-clinical isolates of *E. coli* collected in 2009 from Tripoli and Benghazi hospitals. This study was also conducted to asses the incidence of $bla_{\text{CTX-M}}$ group1 encoding gene along with the mobile genetic element IS*Ecp1* that facilitates its movement and expression.

The results of this section describe the incidence of *E. coli* collected from clinical settings from Tripoli and Benghazi, it also demonstrates the prevalence of ESBLs among these isolates, particularly of CTX-M group1 type. This section provides an evidence of the occurrence of chromosomally and plasmid mediated CTX-M-15 and CTX-M-3 in association with the insertion sequence IS*Ecp1*.

4.2 Results

4.2.1 Characterisation of *E. coli* isolates and antimicrobial susceptibility testing

Thirty nine isolates of *E. coli* were collected in a 4 week period in 2009 from patients admitted to different wards and ICUs from 10 hospitals in Tripoli and Benghazi (Table C.1). Some of the isolates were also from hospital environments such as mechanical ventilators, floors, walls, bedsides and other parts of the hospitals (see Appendix C). The MIC_{50} and MIC_{90} values are shown in table 4.1. Ceftazidime showed higher MIC_{50} and MIC_{90} than that of cefotaxime, low MIC50 and MIC90 was observed for carbapenems whereas high range was shown for piperacillin/tazobactam and ampicillin. In general, high-level of resistance was observed towards 3rd generation cephalosporins. Twenty four out of 39 (61.5 %) were resistant to cefotaxime, 16/39 (41%) resistant to cefuroxime and 17/39 (43.5%) were resistant to ceftazidime. Few of the isolates (7/39) (17.9%) were resistant to ciprofloxacin and 2/39 (5%) and to piperacillin-tazobactam. Those isolates displaying resistance to 3rd generation cephalosporins also showed resistance to aztreonam, trimethoprim sulphamethoxazole - 53.8%, and 35.8% respectively.

4.2.2 Detection of TEM, SHV and CTX-M type ESBL genes

Amplification of bla_{TEM} and bla_{SHV} has shown the occurrence of bla_{TEM} in 7 isolates and bla_{SHV} in 8 *E. coli* isolates tested. Amplification of the major CTX-M groups (1, 2 ,8 ,9 and 26) showed that 23 out of 39 (58.9%) were

positive for CTX-M group 1 and only one *E. coli* isolate gave PCR product for the CTX-M group 9 (Figures 4.1&4.2). The other CTX-M groups were negative. The association of the insertion sequence, IS*Ecp1*, with $bla_{\text{CTX-M-15}}$ occurred in all cases where CTX-M group 1 was present (Figures 4.3&4.4). Moreover, three isolates; AES226, AES228 & AES232 showed the occurrence of an additional CTX-M group 1 gene. Sequencing these PCR products showed the association of $bla_{\text{CTX-M}}$ group1 with IS*Ecp1* in 22/26 (84.62%). The sequencing results of the three different PCR products obtained at 620bp (isolates; AES226, AES228 & AES232) were positive for CTX-M-3 in association with IS*Ecp1* in addition to the CTX-M-15/IS*Ecp1* also carried by these strains. Sequencing results of the single PCR product from the CTX-M group 9 showed the occurrence of CTX-M-19. Interestingly, a deletion event has been detected in the insertion sequence located adjacent to $bla_{\text{CTX-M-15}}$. This deletion event has been found in some insertion sequences, it shows that the IS*Ecp1* is occasionally not intact and probably played a role in the movement of $bla_{\text{CTX-M}}$ group1 with some *E. coli* isolates (Figure 4.5 & 4.6).

4.2.3 Transconjugation experiments

A subset (n=20) of the CTX-M positive *E. coli* were used to study the plasmids carrying the CTX-M-15 genes. The results of the transconjugation experiments using the GFP *E. coli* as a recipient showed that transconjugation was observed in 19 out of 20 (95%). Antibiotic resistance profile of *E. coli* transconjugants, AES224T, AES226T, AES228T and AES231 showed the

occurrence of virtually the same resistance profile from parents to transconjugants (Table 4.2). Ceftazidime resistant transformants were confirmed by PCR. Transconjugation was also conducted on the *E. coli* isolate positive for CTX-M-19. The plasmid carrying $bla_{\text{CTX-M-19}}$ was able to move to the recipient *E. coli* conferring ceftazidime which was further confirmed by PCR.

4.2.3.1 Antibiotic Resistance profile of *E. coli* CTX-M transconjugants

Antibiotic resistance profile of *E. coli* transconjugants; AES224T, AES226T, AES228T, and AES231T are virtually the same as their donor strains. The original GFP *E. coli* strain is fully sensitive a part of rifampicin; subsequently mating *E. coli* with GFP *E. coli* (recipient) indicates the movement of antibiotic resistance mechanism from parents to transconjugants via conjugative plasmids. The resultant GFP *E. coli* were resistant to aminoglycosides, aztreonam, ampicilin, amoxicillin/clavulanate, β-lactam antibiotics such as cephalosporins, and third generation cephalosporins, they were sensitive to carbapenems and monobactams. (Table 4.2).

Table 4.1. MIC_{50} and MIC90 of *E. coli* isolates

Antibiotic	MIC_{50}	MIC_{90}	Range
Ceftazidime	16	32	4 – 32
Cefotaxime	8	64	2 – 64
Imipenem	0.5	1	0.125 – 1
Meropenem	0.5	1	0.125 – 1
Aztreonam	16	16	8 – 16
Piperacillin/Tazobactam	8	128	4 – 128
Ciprofloxacin	2	4	0.5 – 8
Ampicillin	16	64	4 – 64
Gentamicin	16	16	2 – 16

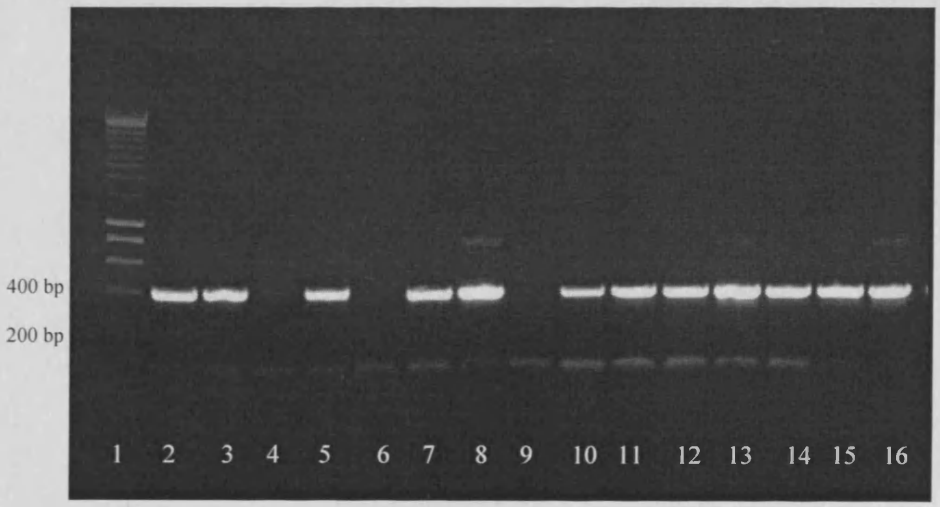

Figure 4.1 Multiplex PCR to detect CTX-M groups; 1, 2, 8, 9 & 26 in *E. coli*. Lane1: Marker. Lane2: *E. coli* AES11. Lane3: *E. coli*AES35. Lnae4: *E. coli* AES58. Lane5: *E. coli* AES120. Lnae6: *E. coli* AES128. Lane7: *E. coli* AES195. Lane8: *E. coli* AES202. Lane9: *E. coli* AES212. Lane10: *E. coli* AES224. Lne11: *E. coli* AES226. Lane12: *E. coli* AES227. Lane13: *E. coli* AES228. Lnae14: *E. coli* AES230. Lnae15: *E. coli* AES231. Lane16: *E. coli* AES232.

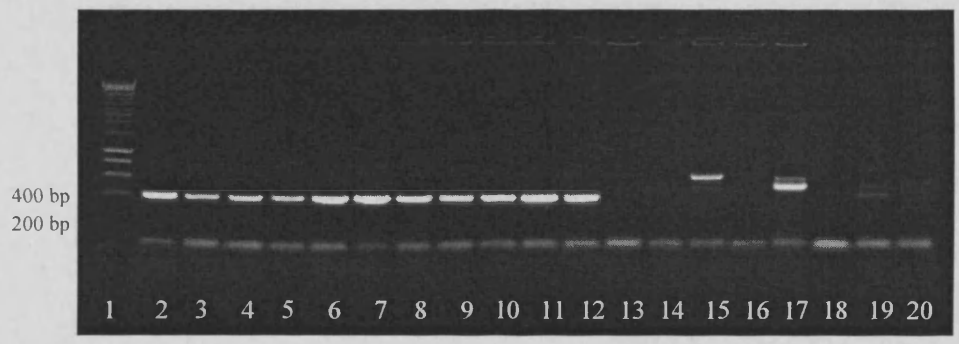

Figure 4.2 Multiplex PCR to detect CTX-M groups; 1, 2, 8, 9 & 26 in *E. coli*. Lane1: Marker. Lane2: *E. coli* AES237. Lane3: *E. coli* AES239. Lane4: *E. coli* AES240. Lane5: *E. coli* AES243. Lane6: *E. coli* AES244. Lnae7: *E. coli* AES245. Lnae8: *E. coli* AES246. Lane9: *E. coli* AES247. Lane10: *E. coli* AES248. Lane11: *E. coli* AES262. Lane12: *E. coli* AES101. Lane13: *E. coli* AES922. Lane14: *E. coli* AES932. Lane15: *E. coli* AES937. Lane16: *E. coli* AES938. Lane17: *E. coli* AES941. Lane18: *E. coli* AES944. Lnae19: *E. coli* AES962. Lane20: *E. coli* AES964

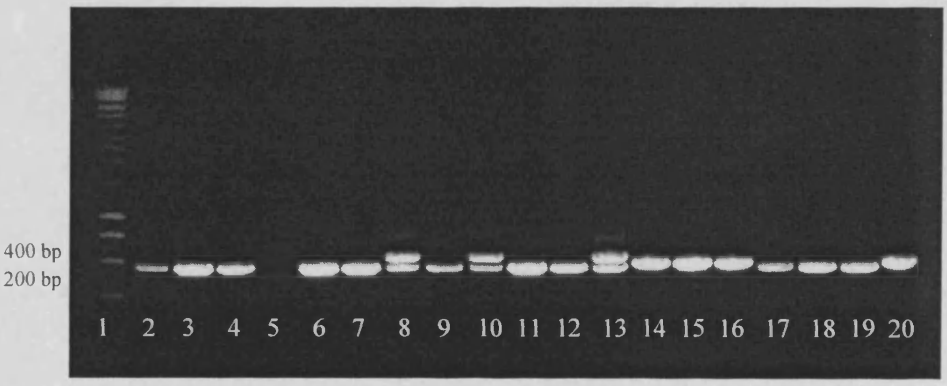

Figure 4.3 Detection of $bla_{\text{CTX-M}}$ group1 and IS*Ecp1* in *E. coli*. Lane1: Marker. Lane2: *E. coli* AES11. Lane3: *E. coli*AES35. Lnae4: *E. coli* AES120. Lane5: *E. coli* AES195. Lnae6: *E. coli* AES202. Lane7: *E. coli* AES224. Lane8: *E. coli* AES226. Lane9: *E. coli* AES227. Lane10: *E. coli* AES228. Lne11: *E. coli* AES230. Lane12: *E. coli* AES231. Lane13: *E. coli* AES232. Lnae14: *E. coli* AES237. Lnae15: *E. coli* AES239. Lane16: *E. coli* AES240. Lane17: *E. coli* AES243. Lane18: *E. coli* AES244. Lane19: *E. coli* AES245. Lane20: *E. coli* AES246.

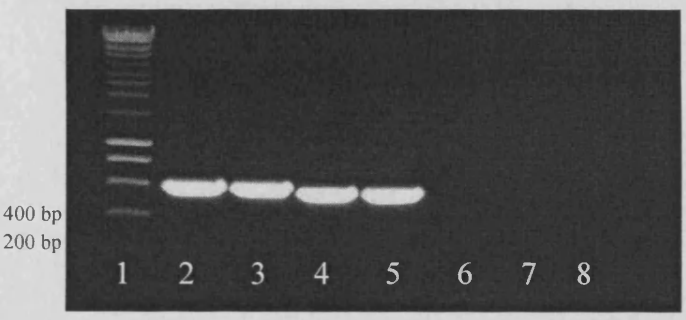

Figure 4.4 Detection of *bla*$_{CTX-M}$ group1 and IS*Ecp1* in *E. coli*. Lane1: Marker. Lane2: *E. coli* AES247. Lane3: *E. coli*AES248. Lnae4: *E. coli* AES262. Lane5: *E. coli* AES101. Lnae6: *E. coli* AES937. Lane7: *E. coli* AES941. Lane8: *E. coli* AES1006.

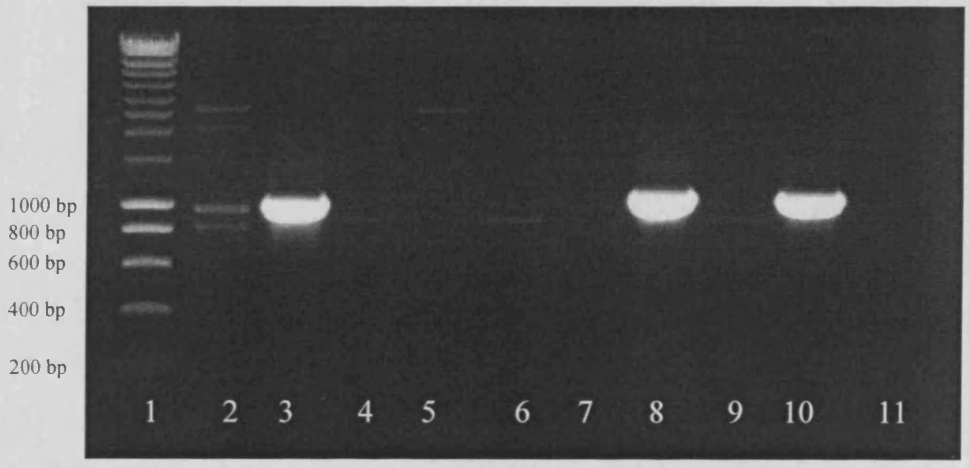

Figure 4.5 Detection of *bla*$_{CTX-M}$ group1 in association with an intact copy of IS*Ecp1* in *E. coli*. Lane1: Marker. Lane2: *E. coli* AES11. Lane3: *E. coli*AES35. Lnae4: *E. coli* AES120. Lane5: *E. coli* AES195. Lnae6: *E. coli* AES202. Lane7: *E. coli* AES224. Lane8: *E. coli* AES226. Lane9: *E. coli* AES227. Lane10: *E. coli* AES228. Lne11: *E. coli* AES230.

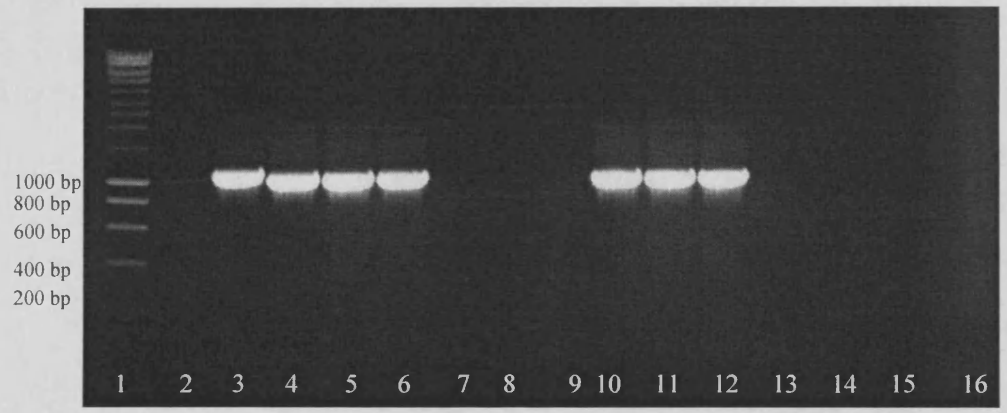

Figure 4.6 Detection of *bla*$_{CTX-M}$ group1 in association with IS*Ecp1* in *E. coli*. Lane1: Marker. Lane2: *E. coli* AES231. Lane3: *E. coli* AES232. Lane4: *E. coli* AES237. Lane5: *E. coli* AES239. Lane6: *E. coli* AES240. Lnae7: *E. coli* AES243. Lane8: *E. coli* AES244. Lnae9: *E. coli* AES245. Lane10: *E. coli* AES246. Lane11: *E. coli* AES247. Lane12: *E. coli* AES248. Lane13: *E. coli* AES262. Lane14: *E. coli* AES101A. Lane15: *E. coli* AES937. Lane16: *E. coli* AES941

The results of the amplification of *bla*$_{CTX-M}$ group1 and IS*Ecp1* to detect the occurrence of the full sequence of the insertion sequence IS*Ecp1* showed that in 12 out of 22 (54.5%) of isolates a deletion event is occurred in the insertion sequence, The results also demonstrated that 10 out 22 (45.4%) had the full sequence of IS*Ecp1*. Amplification of *bla*$_{CTX-M}$ group1 and IS*Ecp1* genes in the transconjugants GFP showed that 18 out of 20 (90%) showed the occurrence of both genes.

Table 4.2 Sensitivity profile of *E. coli* parents and transconjugants

Antibiotic	AES224	AES224T	AES226	AES226T	AES228	AES228T	AES231	AES231T
Amikacin	S	16	S	16	S	16	S	16
Ampicillin	>8	>8	>8	>8	>8	>8	>8	>8
Aztreonam	>16	>16	>16	>16	>16	>16	>16	>16
Cefotaxime	>4	>4	>4	>4	>4	>4	>4	>4
Ceftazidime	>16	>8	16	>8	16	>8	16	>8
Cefuroxime	>16	>8	>16	>8	>16	>8	>16	>8
Ciprofloxacin	>2	S	S	S	S	S	S	S
Gentamicin	S	>4	>8	>4	>8	>4	>8	>4
Imipenem	S	S	S	S	S	S	S	S
Meropenem	S	S	S	S	S	S	S	S
Nitrofurantoin	S	S	S	S	S	S	S	S
Piperacillin/ Tazobactam	S	S	S	S	S	S	S	S
Trimethoprim	-	S	-	S	-	S	-	S
Trimethopri/ Sulphamethoxazole	S	S	S	S	S	S	S	S
Amoxicillin/ clavulanate	16	16	16	16	16	16	16	16

T: Transconjugants

4.2.4 Plasmid typing of ESBL positive *E. coli* isolates

Typing of a subset of $bla_{CTX\text{-}M}$ group1 positive *E. coli* isolates by PCR to identify the plasmids responsible for the carriage and movement of CTX-M group1 and IS*Ecp1* showed that more than one type of plasmids has been detected in some these isolates. AES224 was positive for incFIA, AES226, and its transconjugant were found positive for IncFII. AES237 and its

transconjugant AES237T were carrying *bla*$_{CTX-M}$ group1 on IncI plasmid. AES243 was detected positive for IncF plasmid.

4.2.5 Detection of plasmid mediated *bla*$_{CTX-M}$ group1 and IS*Ecp1* genes in parents and transconjugants of *E. coli*

PFGE of *S*1 digests of a subset of the whole genomic DNA of parents and transconjugants of *E. coli* are shown in figures (4.7&4.9). Probing of PFGE gels of figures 4.7&4.9 with *bla*$_{CTX-M-15}$ is illustrated in figures (4.8&4.10). These results demonstrated the incidence of one copy of *bla*$_{CTX-M}$ group1 in parents of *E. coli* isolates; the results of probing provide an evidence of the movement of *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M}$ group1 from parents to transconjugants. During conjugation, on occasions the plasmid carrying *bla*$_{CTX-M-15}$ changed in size. *bla*$_{CTX-M-15}$ has been detected on a plasmid with a size of 100 kb in three of the parents; AES226, AES228 & AES232 and on 100 kb in *bla*$_{CTX-M}$ group1, AES35, AES227, and AES231; however, during conjugation *bla*$_{CTX-M}$ group1 was detected on two plasmids (100 and 350 kb) in 3 of the transconjugants (AES226T, AES228T & AES232T) and on 100 and 350 kb of the transconjugants AES227T and AES231T and on a plasmid of 300kb in transconjugant AES35T. These data show that *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M}$ group1 genes have moved either from one plasmid to another larger plasmid during conjugation or that during the conjugation process the plasmid has acquired chromosomal DNA or two plasmids (one containing *bla*$_{CTX-M}$ group1 gene) have become co-integrative. *bla*$_{CTX-M}$ group1 was also located on a 125kb

plasmid in the donor AES237 as well as its corresponding transconjugant, in one donor (AES224) and its transconjugants bla_{CTX-M} group1 is present on a 175kb plasmid.

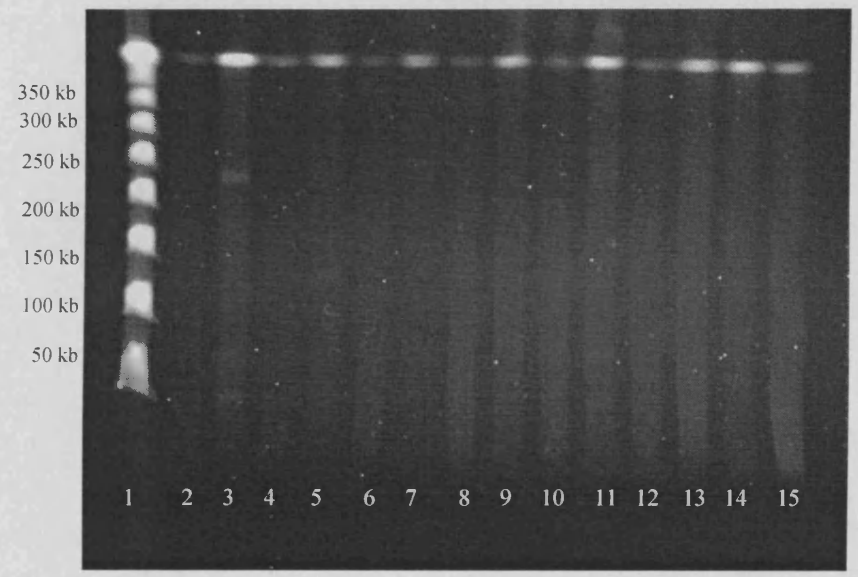

Figure 4.7 PFGE of *S*1 digestion of *E. coli* parents and transconjugants. Lane1: Marker, Lane2: *E. coli* isolate AES35. Lane3: *E. coli* AES35T. Lane4: *E. coli* AES224. Lane5: *E. coli* AES224T. Lane6: *E. coli* AES226. Lane7: *E. coli* AES226T. Lane8: *E. coli* AES227. Lane9: *E. coli* AES227T. Lane10: *E. coli* AES228. Lane11: *E. coli* AES228T. Lane12: *E. coli* AES231. Lane13: *E. coli* AES231T. Lane14: *E. coli* AES237. Lane15: *E. coli* AES237T.

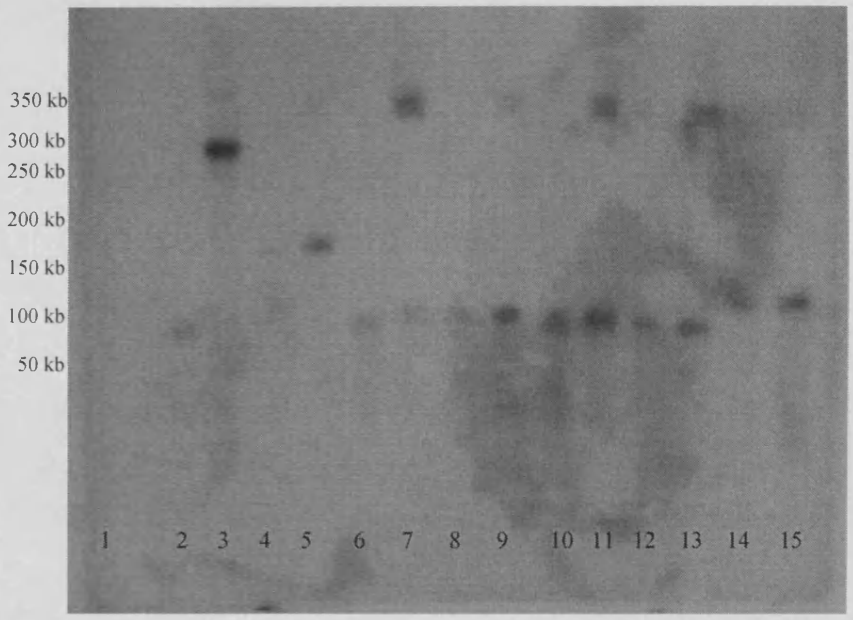

Figure 4.8 Autorad of *E.coli* parents and transconjugants after probing of PFGE gel from fig.4.7 with $bla_{\text{CTX-M-15}}$. Lane1: Marker, Lane2: *E. coli* AES35. Lane3: *E. coli* AES35T. Lane4: *E. coli* AES224. Lane5: *E. coli* AES224T. Lane6: *E. coli* AES226. Lane7: *E. coli* AES226T. Lane8: *E. coli* AES227. Lane9: *E. coli* AES227T. Lane10: *E. coli* AES228. Lane11: *E. coli* AES228T. Lane12: *E. coli* AES231. Lane13: *E. coli* AES231T. Lane14: *E. coli* AES237. Lane15: *E. coli* AES237T.

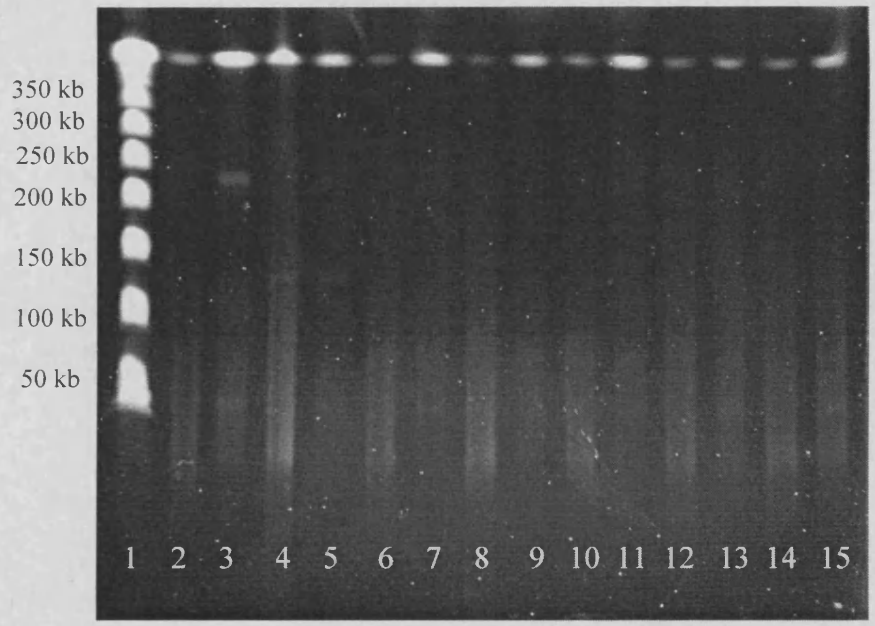

Figure 4.9 PFGE of *S*1 digestion of *E. coli* parents and transconjugants. Lane1: Marker, Lane2: *E. coli* isolate AES35. Lane3: *E. coli* AES35T. Lane4: *E. coli* AES224. Lane5: *E. coli* AES224T. Lane6: *E. coli* AES226. Lane7: *E. coli* AES226T. Lane8: *E. coli* AES227. Lane9: *E. coli* AES227T. Lane10: *E. coli* AES228. Lane11: *E. coli* AES228T. Lane12: *E. coli* AES231. Lane13: *E. coli* AES231T. Lane14: *E. coli* AES232. Lane15: *E. coli* AES232T.

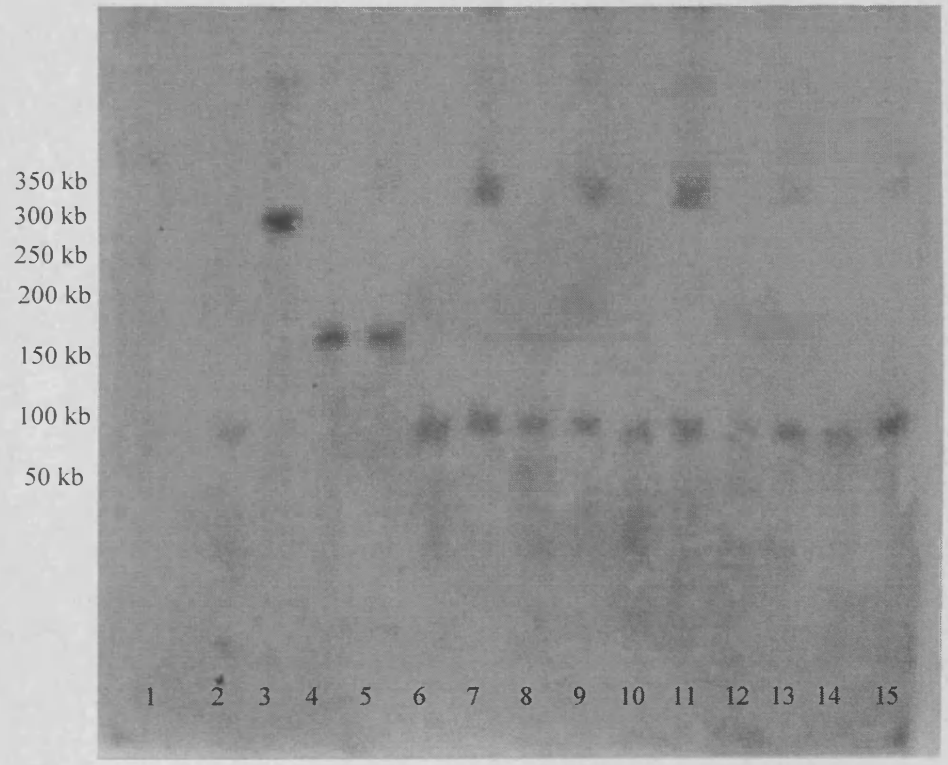

Figure 4.10 Autorad of *E.coli* parents and transconjugants after probing of the PFGE gel from fig. 4.9 with $bla_{CTX\text{-}M\text{-}15}$/IS*Ecp*1. Lane1: Marker, Lane2: *E. coli* AES35. Lane3: *E. coli* AES35T. Lane4: *E. coli* AES224. Lane5: *E. coli* AES224T. Lane6: *E. coli* AES226. Lane7: *E. coli* AES226T. Lane8: *E. coli* AES227. Lane9: *E. coli* AES227T. Lane10: *E. coli* AES228. Lane11: *E. coli* AES228T. Lane12: *E. coli* AES231. Lane13: *E. coli* AES231T. Lane14: *E. coli* AES232. Lane15: *E. coli* AES232T.

4.2.6 Typing of *E. coli* isolates

PFGE of *Xba*1 digests of *E. coli* isolates; AES35, AES224, AES228, AES231, AES232, AES237, AES240, AES243, AES245, AES246, AES247, AES226, AES227, AES11, AES202, AES230, AES239, AES244, AES248 & AES262 are shown in (Figures 4.11 and 4.13). The dendrogram of the PFGE pictures analysis are illustrated in (Figures 4.12 and 4.14). These results showed the incidence of 3 groups of clones among the 20 *E. coli* isolates examined. One clonal group, isolates AES226, AES227, AES228, AES232 and AES231, were clinical and hospital environmental isolates from an ICU as part of a screen from the ICU of the Paediatric hospital in Benghazi, these isolates were slightly different with computer analysis. AES226 and AES232 were urine samples cultured from two patients admitted to Benghazi peadiatric hospital whereas, AES227, AES228 and AES231 were cultured from non-clinical swabs collected from the ICU of the same hospital. *E. coli* isolates; AES243 and AES245 were also clonal and found in urine samples from two different patients suggesting either a dominat Libyan clone or cross-infection. Another two isolates, AES237, AES240, AES246 and AES247 were also clonal despite being dissimilar by dendrogram. Isolates AES237 and AES 246 were from urine samples, while isolates AES240 and AES247 were collected from the corridor and floor of the ICU at the same hospital, this clone (AES237/ AES240) shared more than 90% similarity with isolate AES247 that was cultured from the floor of the same ICU. Isolate AES35 was unrelated to the

other strains isolated from environmental swabs of the same ICU at the Al-Jamhoryia hospital, Benghazi.

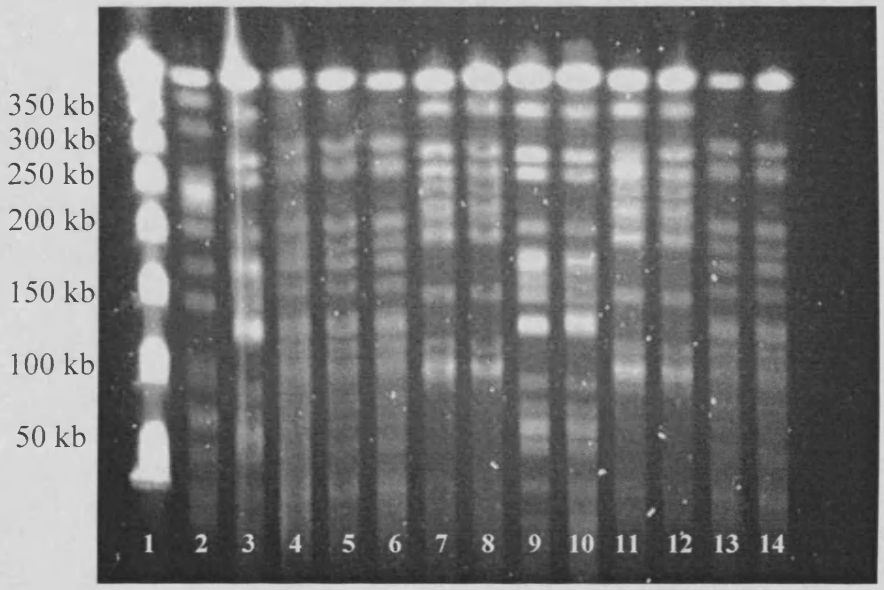

Figure 4.11 PFGE of *Xba*I digestion and separation of genomic DNA according to size. Lane 1: Marker. Lane 2: *E. coli* AES35. Lane 3: *E. coli* AES224. Lane 4: *E. coli* AES228. Lane 5: *E. coli* AES231. Lane 6: *E. coli* AES232. Lane 7: *E. coli* AES237. Lane 8: *E. coli* AES240. Lane 9: *E. coli* AES243. Lane 10: *E. coli* AES245. Lane 11: *E. coli* AES246. Lane 12: *E. coli* AES247. Lane 13: *E. coli* AES226. Lane 12: *E. coli* AES227

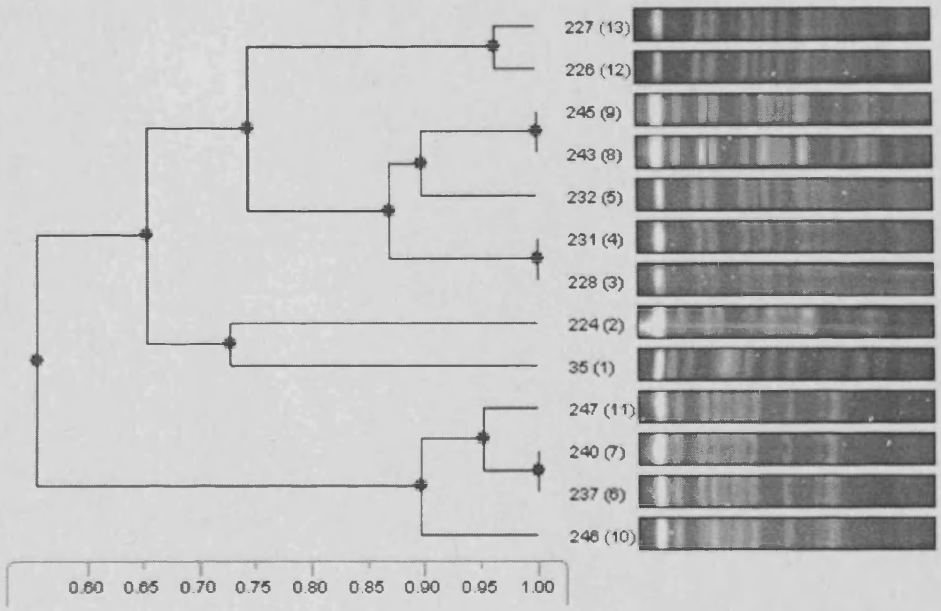

Figure 4.12 Dendrogram of PFGE picture of *E. coli* isolates fig (4.11). Lane 1: Marker. Lane 2: *E. coli* AES35. Lane 3: *E. coli* AES224. Lane 4: *E. coli* AES228. Lane 5: *E. coli* AES231. Lane 6: *E. coli* AES232. Lane 7: *E. coli* AES237. Lane 8: *E. coli* AES240. Lane 9: *E. coli* AES243. Lane 10: *E. coli* AES245. Lane 11: *E. coli* AES246. Lane 12: *E. coli* AES247. Lane 13: *E. coli* AES226. Lane 12: *E. coli* AES227

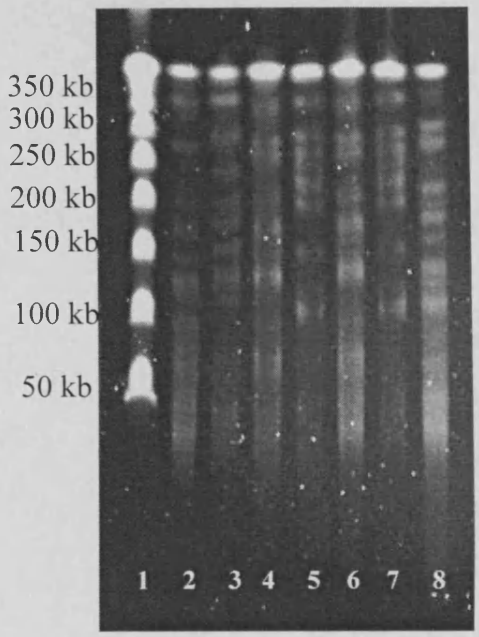

Figure 4.13 PFGE of *Xba*I digestion and separation of genomic DNA according to size. Lane 1: Marker. Lane 2: *E. coli* AES11. Lane 3: *E. coli* iAES202. Lane 4: *E. coli* AES230. Lane 5: *E. coli* AES239. Lane 6: *E. coli* AES244. Lane 7: *E. coli* AES248. Lane 8: *E. coli* AES262.

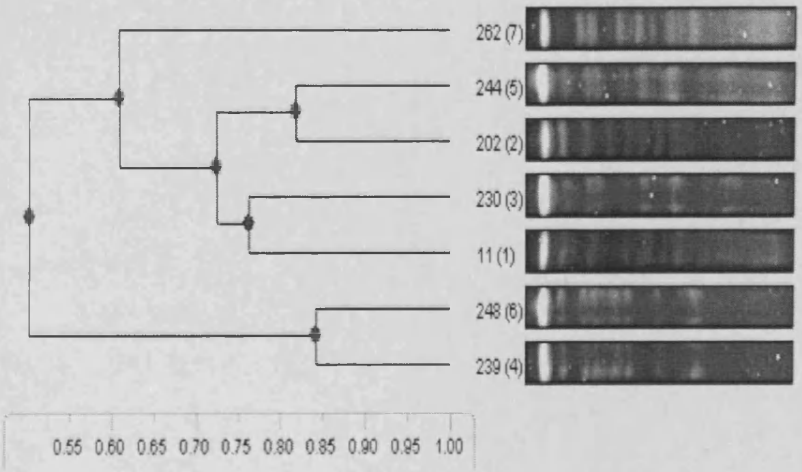

Figure 4.14 Dendrogram of PFGE picture of fig. (4.13). Lane 1: Marker. Lane 2: *E. coli* AES11. Lane 3: *E. coli* iAES202. Lane 4: *E. coli* AES230. Lane 5: *E. coli* AES239. Lane 6: *E. coli* AES244. Lane 7: *E. coli* AES248. Lane 8: *E. coli* AES262.

4.2.7 Detection of chromosomally mediated *bla*$_{CTX-M}$ group1 encoding gene

Probing of the PFGE gels from Figures 4.11 & 4.13 with the radio-labelled *bla*$_{CTX-M-15}$ DNA probe is demonstrated in Figures 4.15 and 4.16. These results show that two copies of the *bla*$_{CTX-M}$ group1 were detected in isolate 11 but only one copy of *bla*$_{CTX-M}$ group1 gene was detected in the other 19 isolates. *bla*$_{CTX-M}$ group1 was found on a 50kb plasmid in 6 isolates (AES35, AES228, AES231, AES232, AES226 and AES227), whereas *bla*$_{CTX-M}$ group1 was carried on a 100kb in isolates; AES237, AES240, AES246, AES247, AES239 and AES248. Four isolates (AES11, AES224, AES243, AES245 and AES230) carry *bla*$_{CTX-M}$ group1 on a plasmid of 125kb. The results in Figures 4.11 and 4.13 showed that isolates; AES35, AES224, AES227, AES231 and AES237 were confirmed to express plasmid mediated CTX-M group1 genes at different plasmid sizes; 50, 125, 50, 50 and 100kb, respectively while isolates AES226, AES228 and AES232 showed plasmid mediated CTX-M group1 genes at 50 kb. The results of probing the PFGE gel of *Xba*I digests provide another evidence of the occurrence of the CTX-M group1 genes on plasmids detected in (Figures 4.8 and 4.10).

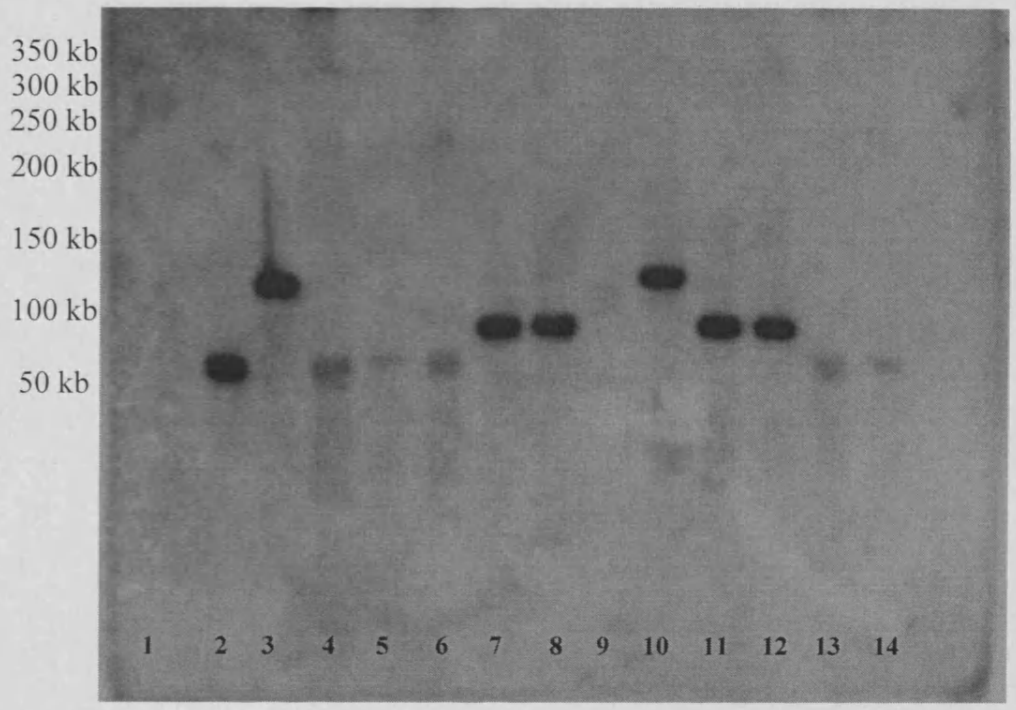

Figure 4.15 Autorad of PFGE gel of fig (4.11) after probing with CTX-M-15. Lane 1: Marker. Lane 2: *E. coli* AES35. Lane 3: *E. coli* AES224. Lane 4: *E. coli* AES228. Lane 5: *E. coli* AES231. Lane 6: *E. coli* AES232. Lane 7: *E. coli* AES237. Lane 8: *E. coli* AES240. Lane 9: *E. coli* AES243. Lane 10: *E. coli* AES245. Lane 11: *E. coli* AES246. Lane 12: *E. coli* AES247. Lane 13: *E. coli* AES226. Lane 14: *E. coli* AES227

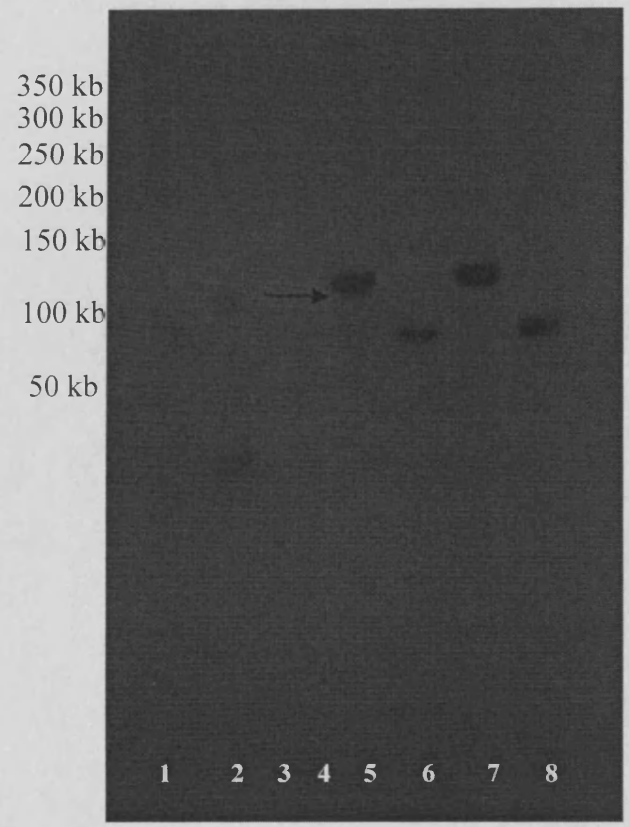

Figure 4.16 Autorad of PFGE gel of fig. (4.13) after probing with CTX-M-15. Lane 1: Marker. Lane 2: *E. coli* AES11. Lane 3: *E. coli* iAES202. Lane 4: *E. coli* AES230. Lane 5: *E. coli* AES239. Lane 6: *E. coli* AES244. Lane 7: *E. coli* AES248. Lane 8: *E. coli* AES262.

4.2.8 Detection of class 1 integrons & Tn*402* type transposons

The results of amplification of class 1 integrons from a subset of 14 isolates of *E. coli* isolates selected according to their resistance to aminoglycosides and trimethoprim, demonstrated that 7 isolates; AES11, AES237, AES240, AES243, AES245, AES246 and AES247 out of 15 yielded PCR products of approx. 2kb. Sequencing of the 2 kb PCR products obtained from isolates; AES11, AES245 and AES247 revealed the presence of a classical class 1 integron. The genetic context of the three integrons were exactly the same containing two gene cassettes; *dfrA*17 and *aadA*5 flanked with the integrase gene (*IntI1)* and the quaternary ammonium compound gene (*qacΔE)*, (Figure 4.17). The integron-positive strains were collected from different sources. Isolates AES11 and AES245 were from urine samples from patients admitted to Al-Jamhoriya hospital and Paediatric hospital in Benghazi, whereas isolate AES247 was from an ICU surface in the Benghazi Paediatric hospital. PCR experiments performed on these isolates to detect the occurrence of Tn*402* type transposons did not detect the desired amplicons.

Figure 4.17 Genetic context of class 1 integrons found in Libyan *E. coli* isolates. IntI1: Integrase gene. dfrA17: Trimethoprim resistance gene. *aadA*5: Aminoglycoside resistance gene. QacEΔ/SuII: Quaternary ammonium compound resistance gene and sulphonamides' resistance gene.

4.3 Discussion

This section describes the molecular characterisation of antibiotic resistance in a random collection of *E. coli* from Libyan hospitals. Data from this collection indicate that the spread of $bla_{\text{CTX-M}}$ group1 along with IS*Ecp1* is well established in Libyan health institutions. The results moreover demonstrate the occurrence of $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-19}}$ among the clinical isolates in addition to bla_{SHV} and bla_{TEM}.

E. coli isolates collected from both Tripoli and Benghazi hospitals in Libya showed that multi-antibiotic resistant isolates were found in Benghazi hospitals, particularly in the Benghazi Paediatric Hospital. Isolates collected from inpatients (urine, blood and pus samples) and hospital environments (mechanical ventilators, baby incubators, surfaces, and bed sites) showed marginally higher rate of resistance to antibiotics, more specifically to third generation cephalosporins. There was no observable difference in the resistance rates of *E. coli* isolates cultured from samples collected from patients and isolates cultured from the hospital environment. MICs of 13 isolates showed marginally higher MIC values toward ceftazidime than cefotaxime, this would argue that there is more than one ESBL has contributed to the resistance mechanism of these isolates. Only 3 isolates displayed higher MICs values against cefotaxime compared with that of ceftazidime, this may be attributed to the occurrence of CTX-M type ESBLs, these findings support the report of (Yu & Cheng, 2004; Abassi *et al.*, 2008).

E. coli isolates screened for the occurrence of ESBLs showed the prevalence of CTX-X-M group 1 and CTX-M group 9 among these isolates. Detailed investigation on this group of CTX-M showed the incidence of $bla_{\text{CTX-M}}$ group1 as the most prevalent ESBL in these isolates. Three isolates demonstrated the occurrence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-3}}$, whereas AES1006 demonstrated the presence of $bla_{\text{CTX-M-19}}$ type ESBLs. $bla_{\text{CTX-M-3}}$ has been detected in three isolates; AES226, AES228 and AES232 in addition to $bla_{\text{CTX-M-15}}$.

The incidence of $bla_{\text{CTX-M}}$ group1 was high (58.9%), this percentage is considered high and may reflect the longstanding antibiotic pressure on cephalosporins in particular 3rd generation cephalosporins. A possible explanation of the widely scattered $bla_{\text{CTX-M}}$ group1 may be attributed to the horizontal gene transfer and/or due to the role of the insertion sequence IS*Ecp1* (Abbassi et *al.*, 2008). This is perhaps not too surprising as several reports showed the global distribution of $bla_{\text{CTX-M-15}}$ in Europe, Asia and Africa (Woodford *et al.*, 2004; Gonullu *et al.*, 2008; Lavollay *et al.*, 2006, Yu & K. Cheng, 2004; Ramdani-Bouguessa, *et al.*, 2006; Abbassi et *al.*, 2008). The findings of this work are in accordance with the records on the dissemination of CTX-M-15 and CTX-M-3 in *E. coli* reported by (Ramdani-Bouguessa, *et al.*, 2006) in Algeria suggesting the enhancement of $bla_{\text{CTX-M-15}}$ movement by IS*Ecp1*.

IS*Ecp1* gene was determined for $bla_{CTX\text{-}M\text{-}15}$ positive *E. coli*; however, a deletion event has been observed by PCR in 12 out of 22 positive isolates to IS*Ecp1*. According to the findings of this work, this deletion event does not seem to affect the movement of CTX-M group1 gene from donor cells to recipients, moreover the IS*Ecp1* either intact or with a deletion event moved with the β-lactamase gene by transconjugation experiments. It is likely to responsible for the movement of $bla_{CTX\text{-}M}$ group1 within the same strain but to different plasmid size as shown in *E. coli* isolates AES226, AES227, AES228, AES230, AES231 and AES232 (Figures 4.8 and 4.10) The mobility and expression of CTX-M type ESBLs by IS*Ecp1* has been proposed by Poirel *et al.*, 2003; Abbassi et *al.*, 2008.

$bla_{CTX\text{-}M}$ group1 and IS*Ecp1* have been detected in donors and transconjugants of *E. coli* on five different plasmid sizes; 100, 175, 300 and 350kb. *E. coli* donors showed the occurrence of $bla_{CTX\text{-}M}$ group1 and IS*Ecp1* on one plasmid for each isolate. In *E. coli* isolates; AES35, AES226, AES227, AES228, AES231 and AES232, $bla_{CTX\text{-}M}$ group1 and IS*Ecp1* were detected on two different plasmid sizes in recipients whereas they were found in one plasmid location in donors. Interestingly, the data from this study shows the fluidity of $bla_{CTX\text{-}M}$ group1 and IS*Ecp1* by mobilising to another plasmid during conjugation. Such events are rarely reported.

Three plasmid types have been detected by PCR in *E. coli* isolates, IncI in AES237, IncFII in AES226 and IncFIA in AES224. Several reports have shown that IncF plasmids (IncFII and IncFIA) are responsible for carrying and facilitating the movement of $bla_{CTX\text{-}M}$ group1 and IS*Ecp1* element. (Gonullu *et al.*, 2008; Villa *et al.*, 2010; Lavollay *et al.*, 2006; Partridge *et al.*, 2011).

Amongst all tested isolates for class 1 integrons, one integron composed of two gene cassettes; *dfr*A17 and *aad*A5 and has been previously described reported from patient suffered from UTI in Australia. The same integron was also reported in Spain and China among *E. coli* isolates (Vinue *et al.*, 2008; Tang *et al.*, 2011).

Typing of *E. coli* isolates was performed on the basis of the incidence of ESBLs more specifically $bla_{CTX\text{-}M}$ group1 among these isolates, this typing resulted in the occurrence of 3 clonal groups clone 1 (AES226, AES227, AES228, AES231 and AES232), clone 2 (AES243 and AES245 and clone 3 (AES237, AES240, AES246 and AES247). Members of clone 1 were collected from patients and the hospital environment, members of clone 2 were from urine samples from two different patients admitted to the same hospital whereas members of clone 3 from two different locations; isolate AES237 and AES246 were from a urine samples while isolate AES240 and AES247 were from the hospital environment of the same hospital. These findings would suggest that the inter-dissemination of clonal isolates of *E. coli*

in the same hospital is due to longstanding problem and propose earlier establishment of the gene pool in this hospital. Clonal dissemination of *bla*$_{CTX-M}$ group1 in *E. coli* has grasped the attention of many investigators to understand the epidemiology of antibiotic resistance in the clinical settings and even outside the hospitals to study the contribution of clonal isolates in the community (Lavollay *et al.,* 2006; Mashana *et al.*, 2011). The findings of this section are in accordance to somewhat with clonally spread of *E. coli* strains harbouring plasmid mediated *bla*$_{CTX-M-15}$ genes reported by (Coque *et al.*, 2008).

Chapter Five

Detection of *bla*$_{\text{VIM-2}}$ in *P. aeruginosa* from Benghazi

5.1 Introduction

P. aeruginosa is capable of causing internal and external infections to humans and largely linked with CAIs and HAIs. It contributes by 10 % among all other bacterial infections in hospitals and is considered as the leading cause of cross infections; VAP and wound infections (Enoch *et al.*, 2007). *P. aeruginosa* antimicrobial resistance is continuing to rise and this is likely elucidated by the ability of this micro-organism to live in diverse environments and share genetic information with numerous species of bacteria that results in withstanding the effect of antimicrobials by means of antibiotic hydrolysing enzymes in particular MBLs (Walsh *et al.*, 2005), (Gales, *et al.*, 2003). The acquisition of MBLs by *P. aeruginosa* is of particular concern due to the fact that this enzyme confers resistance to all β-lactams with the sole exception of aztreonam. Furthermore, MBL-producing Gram-negative bacteria are resistant to nearly all antibiotics and have become pan-resistant resulting in the wide spread of treatment failure (Pournaras *et al.*, 2003; Yu *et al.*, 2006).

Section 5 deals with the spread of multi-drug resistant isolates of *P. aeruginosa* collected from hospitalised patients, hospital environment swabs in Tripoli and Benghazi. This work focuses on the spread of mobile genetic elements; class 1 integrons and transposons associated with MBLs in 14 *P. aeruginosa* isolates from Libya. The results show the incidence of multi-drug resistant *P. aeruginosa* from clinical and no-clinical sources.

bla$_{VIM-2}$ has been detected in two isolates of *P. aeruginosa* collected from two patients admitted to Al-Jalla hospital in Benghazi. Transconjugation experiments using *E.* coli J53 and *P. aeruginosa* PA01 failed to produce any ceftazidime resistant transformants. *bla*$_{VIM-2}$ has been shown to be chromosomally located in two isolates of *P. aeruginosa.* The investigation did not show the presence of Tn*402* that is usually associated with class 1 integrons to facilitate their mobility. The results also showed the incidence of 3 types of class 1 integrons among 7 isolates of *P. aeruginosa.* Novel integron were submitted to the gene bank and assigned the accession numbers; HE583392.2 and HE583391.2.

5.2 Results

P. aeruginosa collected from clinical and non-clinical samples are illustrated in Table 5.1.

5.2.1 Antibiotic susceptibility testing

Antimicrobial sensitivity testing of 14 clinical and non-clinical isolates of *P. aeruginosa* is shown in Table 5.2. These results show high-level resistance to gentamicin, imipenem, aztreonam, cefotaxime, ceftazidime, ciprofloxacin, piperacillin/tazobactam, trimethoprim/sulphamethoxazole, amoxicillin and ampicillin. *P. aeruginosa* isolates AES30, AES81 AES83 and AES93 had MICs above 8 mg/l. Susceptibility of 10 isolates against amikacin and meropenem was recorded for the other isolates.

5.2.2 Detection of MBLs using Etest

All isolates were subjected to Etest (see section 2.7) using imipenem and imipenem plus inhibitor (IP/IPI) to identify the presence of any MBLs. The results showed that *P. aeruginosa* isolates AES81 and AES83 had high levels of resistance to imipenem yet was sensitive to the presence of EDTA (IPI) and thus indicating the presence MICs higher than 16 mg/l proposing the production of a MBL (Figure 5.1).

5.2.3 Detection of MBL encoding genes

PCR experiments were conducted using primers specific for previously reported MBLs. Data showed the occurrence of 700 bp amplicons from *P.*

aeruginosa isolates AES81 and AES83 (Figure 5.2). The two amplicons resulted from primers designed to amplify bla_{VIM} genes. Both sequences displayed 100% homology to bla_{VIM-2} that is disseminated worldwide (Walsh, *et al.*, 2003).

Table 5.1 List of *P. aeruginosa* used in experiments

P. aeruginosa	Site of collection	Place of collection
AES30	Urine	Al Jamhoryia hospital Benghazi
AES81	Stainless steel container (Chest ward)	Al-Jala hospital Benghazi
AES83	Tip of catheter (ICU)	Al-Jala hospital Benghazi
AES89	Floor of toilet (ICU)	Al-Jala hospital Benghazi
AES91	Suction machine tube (ICU)	Al-Jala hospital Benghazi
AES93	Suction machine outlet	Al-Jala hospital Benghazi
AES146	Floor of toilet (ICU)	Al-Jala hospital Benghazi
AES182	Pus sample	Al-Jala hospital Benghazi
AES273A	Blood sample	Al Jamhoryia hospital Benghazi
AES284	Blood sample	Al Jamhoryia hospital Benghazi
AES287	Urine sample	Al Jamhoryia hospital Benghazi
AES934	Wound infection	Burn and plastic surgery centre Tripoli
AES988	Wound infection	Burn and plastic surgery centre Tripoli
AES998	Wound infection	Burn and plastic surgery centre Tripoli
AES1010	Wound infection	Burn and plastic surgery centre Tripoli

Table 5.2 : Antibiotic sensitivity testing of clinical and non-clinical *P. aeruginosa*

Antibiotic	Sensitive	Resistant
Amikacin	10/15	6/15
Gentamycin	5/15	11/15
Imipenem	7/15	9/15
Meropenem	10/15	6/15
Cefotaxime	2/15	14/15
Ceftazidime	6/15	10/15
Aztreonam	2/15	14/15
Trimethoprime sulphamethoxazole	1/15	15/15
Piperacillin tazobactam	7/15	9/15
Ciprofloxacin	6/15	10/15
Amoxicillin	0/15	15/15
Ampicillin	1/15	15/15

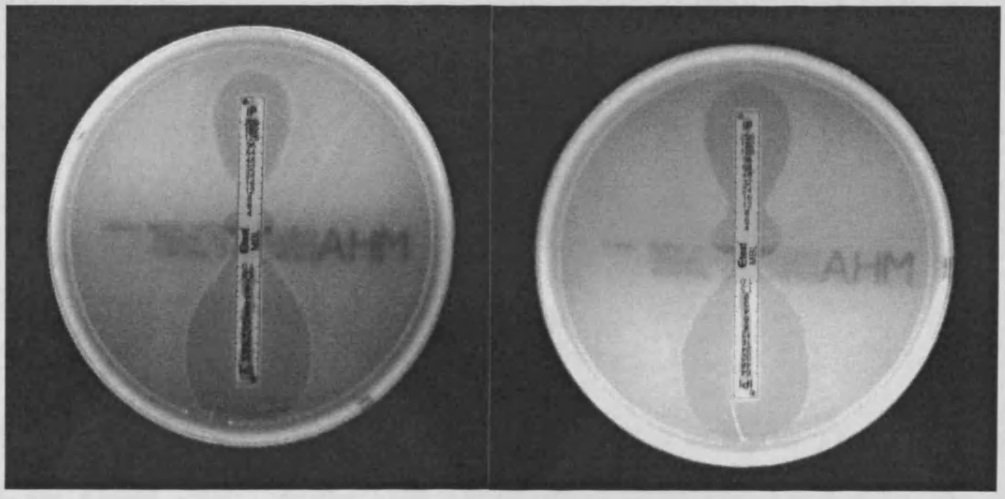

Figure 5.1 Etest of *P. aeruginosa* isolates. A. *P. aeruginosa* AES81. B. *P. aeruginosa* AES83

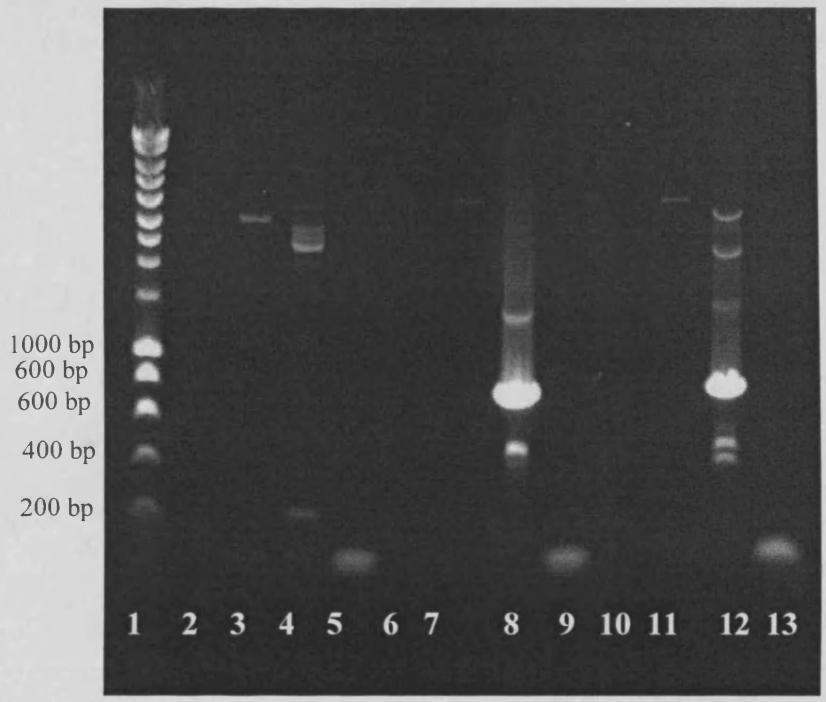

Figure 5.2 Detection of Tn*402* type transposon, Tn*21* and bla_{VIM-2} in *P. aeruginosa* isolates AES81 and AES83. Lane1: Marker. Lane2: Tn*402* in isolate AES81. Lane3: negative control. Lane4: Tn*402* in isolate AES83. Lane5: negative control. Lane6: Tn*21* in isolate AES81. Lane7: negative control. Lane8: bla_{VIM-2} in isolate AES81. Lane9: negative control. Lane10: Tn*21* in isolate AES83. Lane11: negative control. Lane12: bla_{VIM-2} in isolate AES83. Lane13: negative control.

5.2.4 Transconjugation experiments

Transconjugation experiments using J53 and PA01 as recipients were performed to detect the possible occurrence of bla_{VIM-2} on a transferable plasmid. The mating experiments did not produce any ceftazidime resistant *E. coli or P. aeruginosa* transconjugants suggesting that bla_{VIM-2} in these clinical isolates of *P. aeruginosa* is chromosomally located.

5.2.5 Typing of *P. aeruginosa*

PFGE of *Spe*-1 digestion of isolates of *P. aeruginosa* is shown in Figure 5.3. Dendrogram of Figure 5.3&5.7 ; in particular isolates AES30, AES81, AES83, AES89, AES91, AES146, AES182, AES273, AES284 and AES287 are illustrated in Figure 5.4. Typing of *P. aeruginosa* showed that isolates; AES89, AES91, AES93, AES146 and AES182 are clonal despite being collected from two geographically distant places. AES81 was collected from stainless steel container in Chest ward in Al-Jalla hospital in Benghazi whereas AES83 was from a clinical sample from tip of catheter from a patient admitted to the ICU of the same hospital. Isolate AES182 is from a pus sample and isolate 146 is from the floor of an ICU toilet. *P. aeruginosa* isolates AES81 and AES83 are clonal despite the fact that they were from clinical and non-clinical samples.

5.2.6 Detection of chromosomally and plasmid mediated *bla*VIM-2

5.2.6.1 Characterization of chromosomally and plasmid mediated bla_{VIM-2} genes

PFEG of *S*1 genomic DNA digestion of *Pseudomonas* aeruginosa isolates is illustrated in Figure 5.5. Probed PFGE gel of Figures 5.5&5.7 with a custom made probe of bla_{VIM-2} is shown in Figure 5.6&5.8. These results show that bla_{VIM-2} is chromosomally mediated in both isolates positive for the MBL encoding gene, the results of probing of the PFGE gel of *Spe*1 digestion

demonstrate that three copies of bla_{VIM-2} were detected carried by both isolates of *P. aeruginosa* revealing the occurrence of more than one copy on class 1 integrons in each isolate, The bla_{VIM-2} positive isolates were negative for Tn*402* type transposons that might facilitate the mobility of class 1 integrons within the chromosome, intracellular or intercellular but the mechanism of movement of bla_{VIM-2} cannot be attributed to insertion sequences.

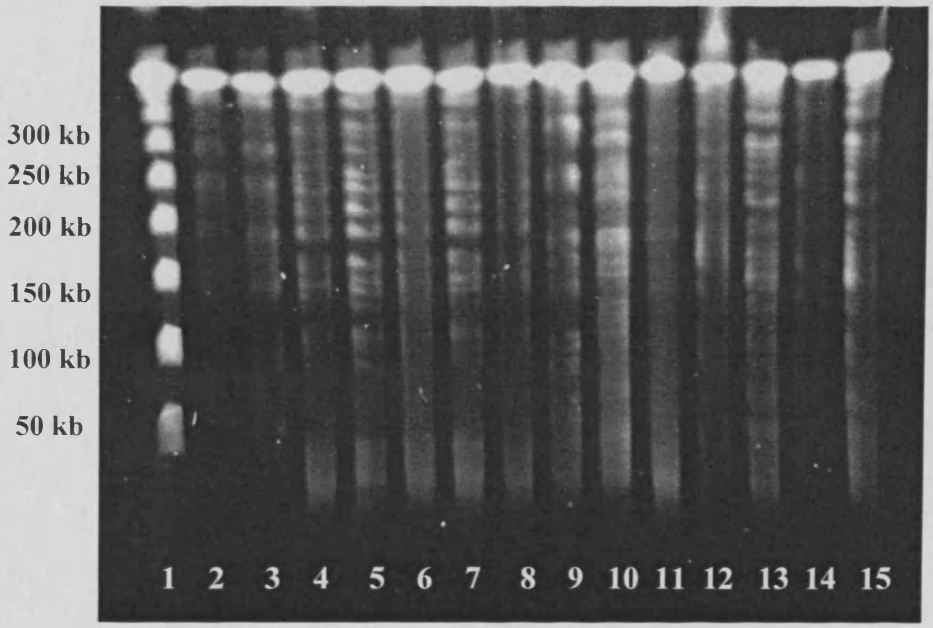

Figure 5.3 PFGE of *Spe*-1 digestion of 14 isolates of *P. aeruginosa*. Lane1: Marker, Lane2: AES81, Lane3: AES83, Lane4: AES89, Lane5: AES91, Lane6: AES93, Lane7: AES146, Lane8: AES182, Lane9: AES273A, Lane10: AES284, Lane11: AES287, Lane12: AES934, Lane13: AES988, Lane14: AES998, Lnae15: AES1010.

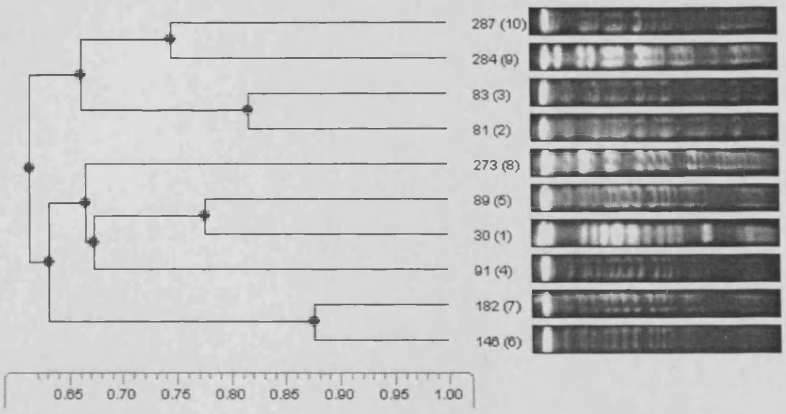

Figure 5.4 Dendrogram of PFGE picture of fig. 5.3 :Lane1: isolate no. 30, Lane2: AES81, Lane3: AES83, Lane4: AES91, Lane5: AES89, Lane6: AES146, Lane7: AES182, Lane8: AES273, Lane9: AES284, Lane10: AES287

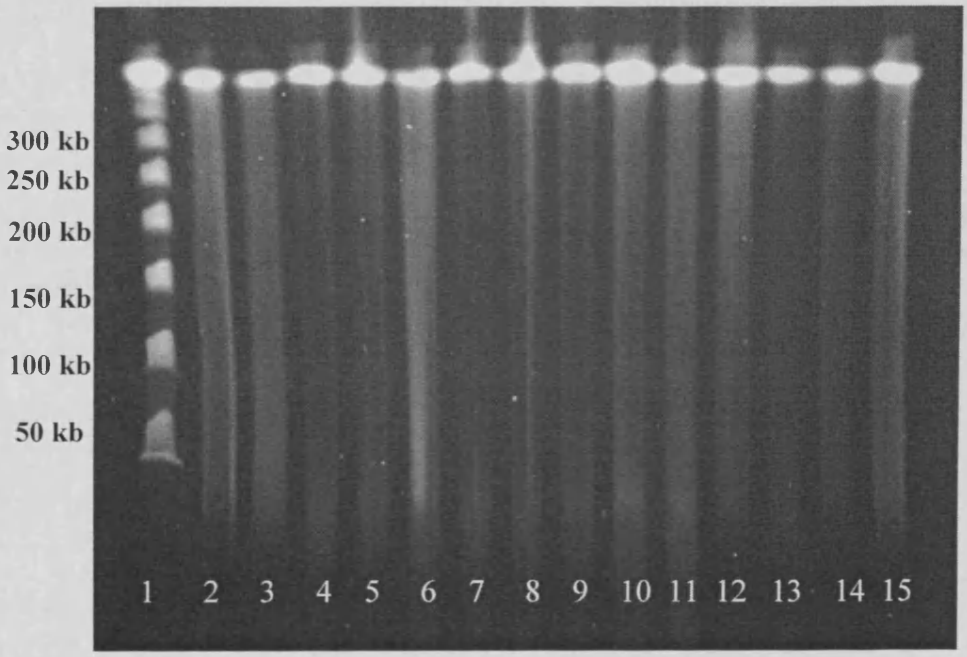

Figure 5.5 PFGE of *S*1 digestion of 14 isolates of *P. aeruginosa*. Lane1: Marker, Lane2: AES81, Lane3: AES83, Lane4: AES89, Lane5: AES91, Lane6: AES93, Lane7: AES146, Lane8: AES182, Lane9: AES273A, Lane10: AES284, Lane11: AES287, Lane12: AES934, Lane13: AES988, Lane14: AES998, Lnae15: AES1010.

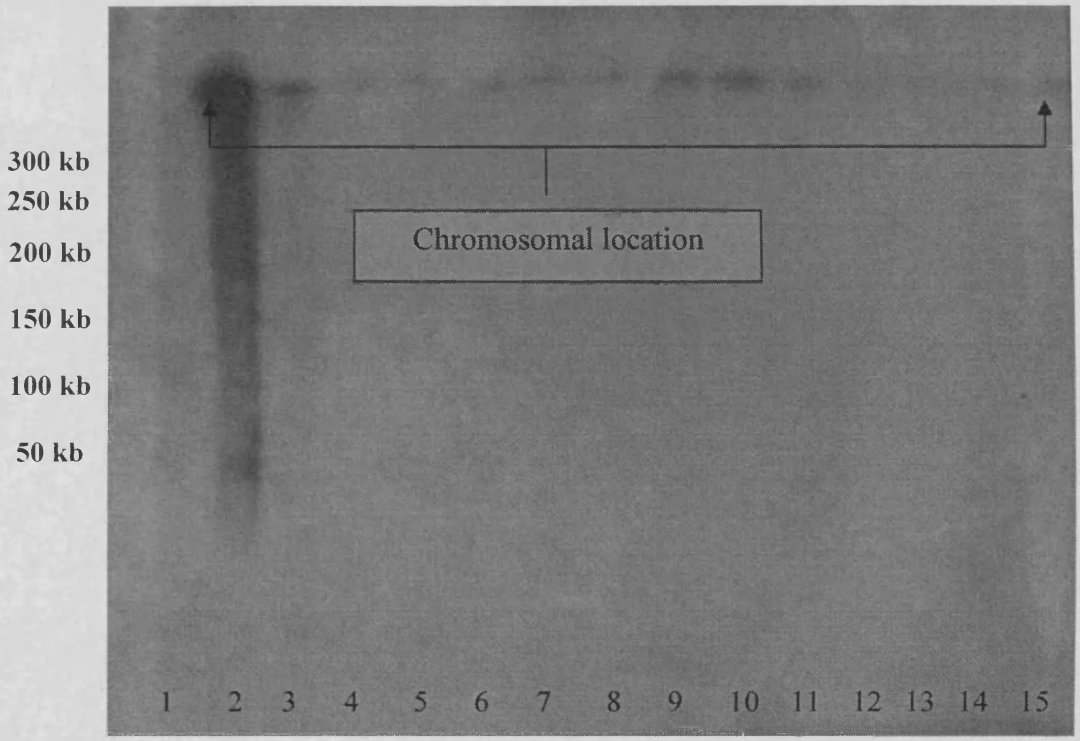

Figure 5.6 Autorad of PFGE of fig. 5.5 after probing wth radio-labelled *bla*$_{VIM-2}$ encoding gene. Lane1: Marker, Lane2: AES81, Lane3: AES83, Lane4: AES89, Lane5: AES91, Lane6: AES93, Lane7: AES146, Lane8: AES182, Lane9: AES273A, Lane10: AES284, Lane11: AES287, Lane12: AES934, Lane13: AES988, Lane14: AES998, Lane15: AES1010.

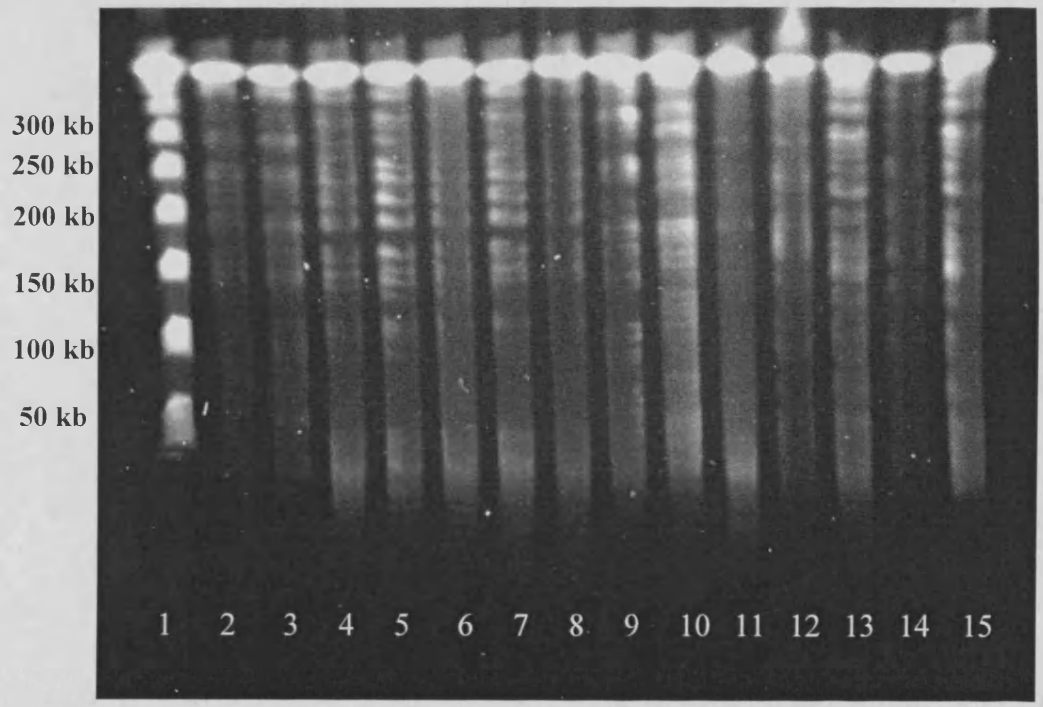

Figure 5.7 PFGE of *Spe*1 digestion of 14 isolates of *P. aeruginosa*. Lane1: Marker, Lane2: AES81, Lane3: AES83, Lane4: AES89, Lane5: AES91, Lane6: AES93, Lane7: AES146, Lane8: AES182, Lane9: AES273A, Lane10: AES284, Lane11: AES287, Lane12: AES934, Lane13: AES988, Lane14: AES998, Lnae15: AES1010.

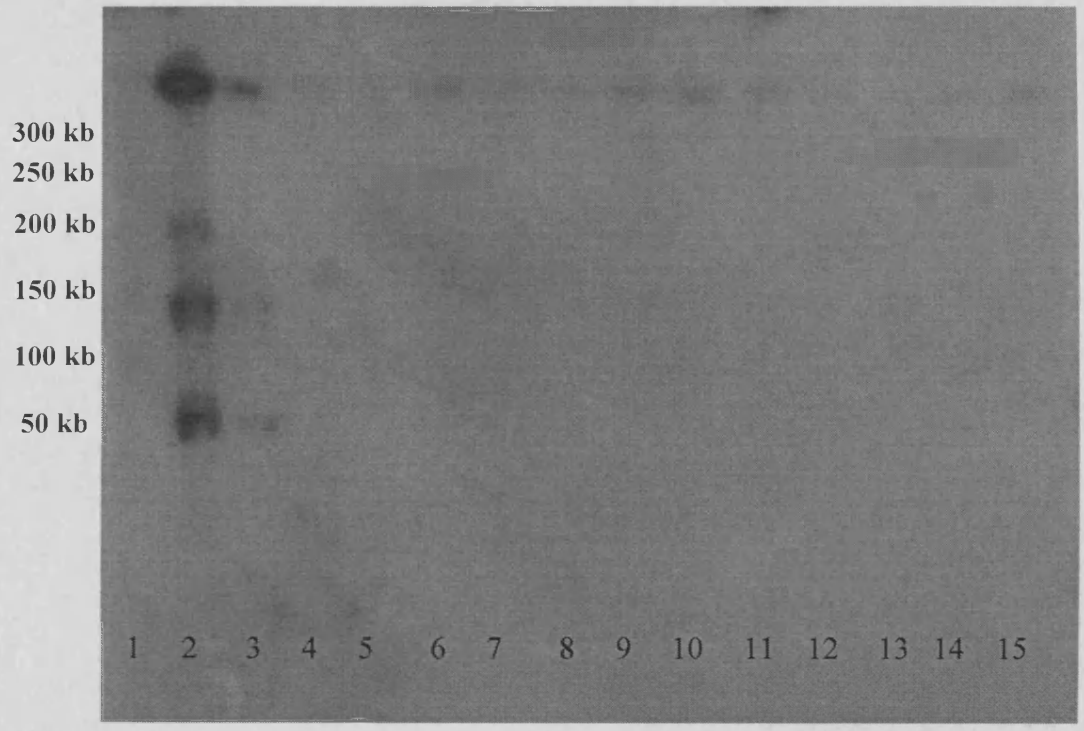

Figure 5.8 Autorad of PFGE of *Spe*1 digestions from fig. 5.7 after probing with radio-labelled bla_{VIM-2} gene. . Lane1: Marker, Lane2: AES81, Lane3: AES83, Lane4: AES89, Lane5: AES91, Lane6: AES93, Lane7: AES146, Lane8: AES182, Lane9: AES273A, Lane10: AES284, Lane11: AES287, Lane12: AES934, Lane13: AES988, Lane14: AES998, Lnae15: AES1010.

5.2.7 Detection of class 1 integrons and transposons

Amplification of class 1 integrons and transposons from clinical and non-clinical *P. aeruginosa* isolates; AES30, AES81, AES83, AES89, AES91, AES93, AES146, AES182, AES273, AES284, AES287, AES934, AES988 and AES1010 showed that 7 out of 15 yielded PCR products for class 1 integrons; moreover, the size of the integrons amplified suggest that more than one gene cassette is involved in each of the integrons. Specifically, PCR amplicons integrons from *P. aeruginosa* isolates AES81, AES83, AES89 and AES182 (Table 5.2) revealed products of sizes 3kb, 2.5kb, 1.5kb and 1.5kb respectively (Figure 5.9). The same integron was found in the two clonal *P. aeruginosa* isolates; AES89 and AES182 and composed of the gene cassette sequence: *intI1, aadA6*, *ORF*, *qacEΔ/sul1* (Figures 5.10C&D) in spite of the different site of collections of these isolates (Table 5.1). Class 1 integrons from AES81 displayed the gene cassette sequence *intI1*, *aad*B, bla_{VIM-2}, *dhfrA*1 *aac6-II*, *qacEΔ/sul1*) (Figure 5.10A). Isolate AES83 possessed the gene cassette sequence *intI1*, aadB1761, *dfrA1*, *aac6-II* and *quacEΔ/sul*1 (Figure 5.10B). PCR on AES83 revealed the occurrence of the same genetic context of the integron detected in AES81 in addition to another novel integron (Figure 5.10B).

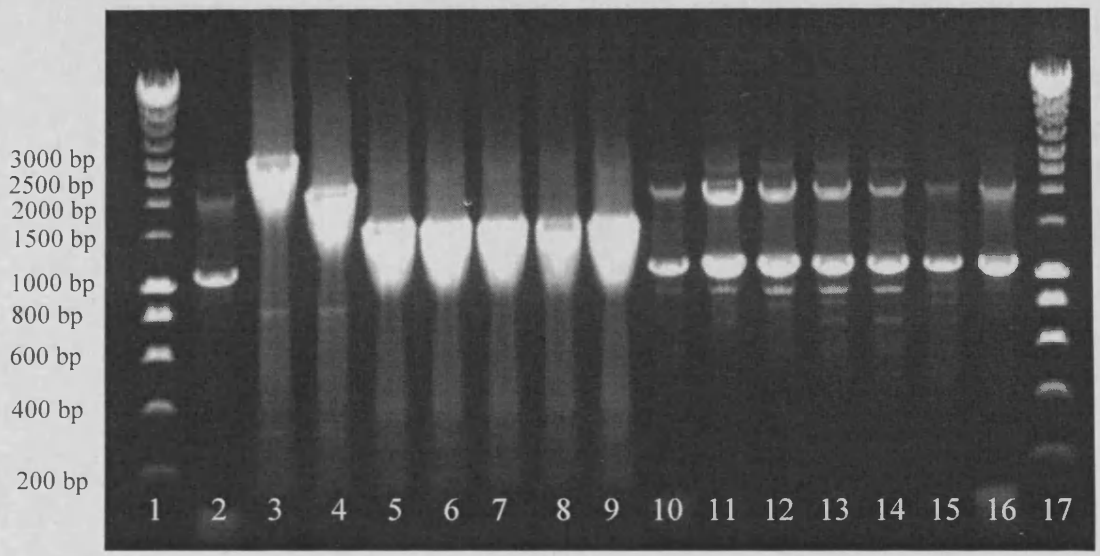

Figure 5.9 Amplification of class 1 integrons from *P. aeruginosa* isolates. Lane1: Marker. Lane2: AES30. Lane3: AES81. Lane4: AES83. Lane5: AES89. Lane6: AES91. Lane7: AES93. Lane8: AES146. Lane9: AES182. Lane10: AES273A. Lane11: AES284. Lane12: AES287. Lane13: AES934. Lane14: AES988. Lane15: AES998. Lane16: AES1010. Lane17: Marker

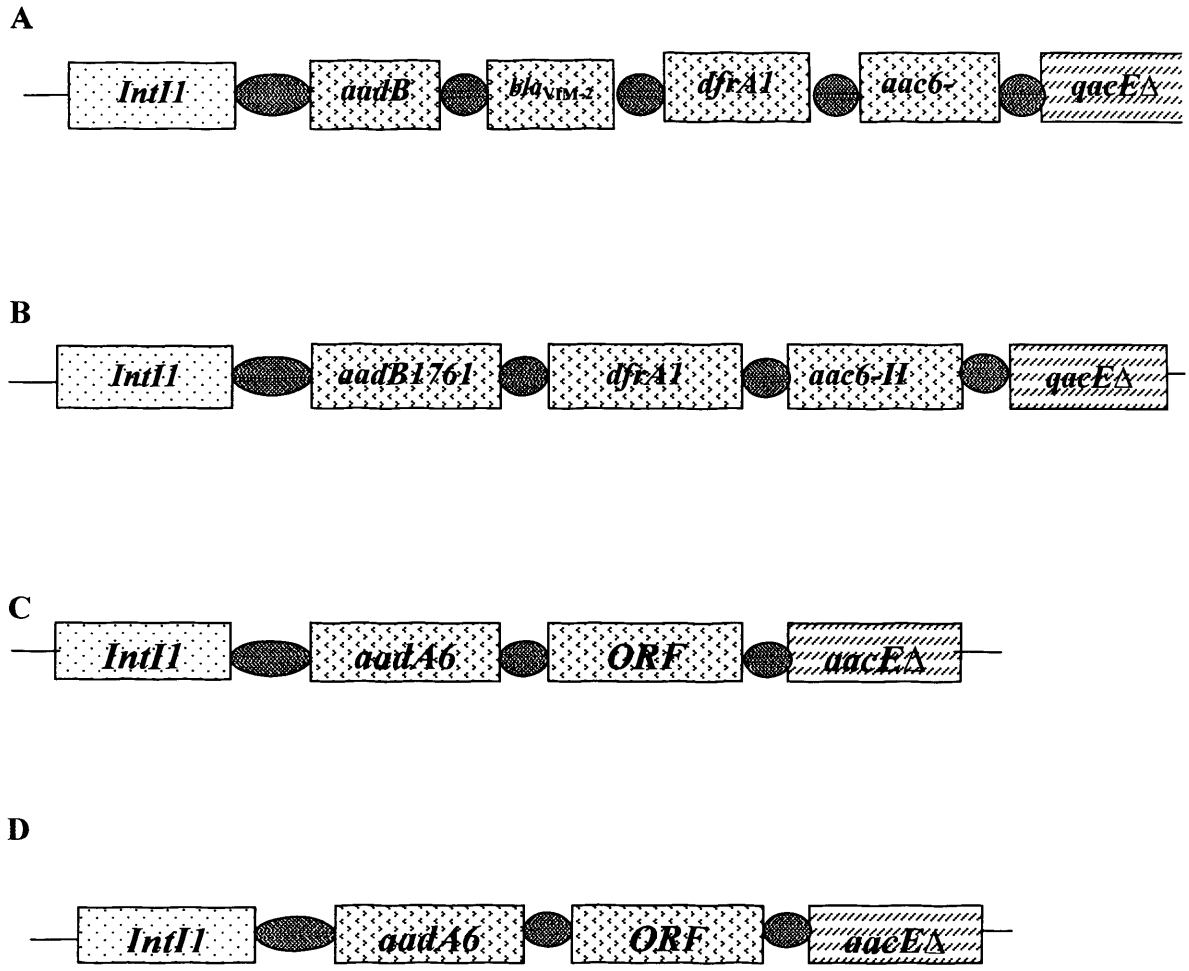

Figure 5.10 Genetic context of class 1 integrons found in Libyan *P. aeruginosa* isolates. A: Class 1 integrons in AES81. B: Class 1 integrons in AES83. C: Class 1 integrons in AES89. D: Class 1 integrons in. AES182. *IntI1*: Integrase gene. aadB: gentamicin resistance gene.. bla_{VIM-2}: Carbapenem resistance MBL gene. *Aac6-II*: aminoglycoside resistance gene, *aadB1761*: gentamicin resistance gene, *dfrA*: trimethoprim resistance gene, *aadA6*: aminoglycoside resistance gene. QacEΔ/SuII: Quaternary ammonium compound resistance gene and sulphonamides' resistance gene.

5.3 Discussion

This section showed the resistance mechanism of some *P. aeruginosa* isolates randomly collected from Tripoli and Benghazi hospitals, the isolates were from clinical and non-clinical samples. Non-clinical samples were taken as there is very little, if any, infection control in these hospitals and it was of interest to see if isolates from environmental swabs matched those causing infections. The Al-Jalla hospital showed the clonal incidence of multi-drug resistant *P. aeruginosa*, these isolates exhibited the occurrence of different class 1 integrons and in some MBL encoding genes.

The overall mechanism of antibiotic resistance in *P. aeruginosa* utilises many antibiotic resistance determinants (Walsh; 2005; Toleman *et al.*, 2007), efflux pumps (Morero *et al.*, 2011; Cabot *et al.*, 2011) and porin alterations (Muller *et al.*, 2011; Tomas *et al.*, 2010). Specific antibiotic resistance determinants can include MBL encoding genes and serine carbapenemases often linked to mobile genetic elements, the former is exemplified by class 1 integrons sometimes associated with Tn*402*-like transposons (Tato *et al.*, 2010; Stokes *et al.*, 2006).

bla_{VIM-2} has been found as gene cassette carried by class 1 integron in *P. aeruginosa* worldwide, in Poland (Toleman *et al.*, 2003), Germany (Valenza *et al.*, 2010), Spain, (Rojo-Bezares *et al.*, 2011), Venezuela (Guevara, A. *et al.*, 2009), United States of America (Aboufaycal *et al.*, 2007) and Ireland (Walsh

& Rogers, 2008). It is also emerging Saudi Arabia (Guerin *et al.*, 2005), Japan (Yatsuyanagi *et al.*, 2004), India and Russia (Toleman *et al.*, 2007) Eastern Europe (Bosnjak *et al.*, 2011; Jovcic *et al.*, 2011) and herein I report the first detection of *P. aeruginosa* positive for bla_{VIM-2} in Libya.

Probing of PFGE gel of *Spe*1 digestion with radio-labelled bla_{VIM-2} indicated that the two clonal isolates positive for the MBL gene had the same gene on the same size DNA fragment. Nevertheless, analysis of the PFGE and subsequent dendrogram demonstrated that the strains are not clonal and share less than 85%. the results of this work are consistent with the studies of Lagatolla *et al.*, 2006 who described the incidence of high-level endemicity of clonally related *P. aeruginosa* carrying bla_{VIM-2}. These finding are dissimilar to the work of Nho *et al.*, 2008 who reported the dissemination of genetically unrelated isolates of *P. aeruginosa* carrying *bla*VIM-2 in Korea. The results are also conflicts somewhat with the findings of Aboufaycal *et al.*, 2007 on the emergence of different ribotypes of *P. aeruginosa* positive for bla_{VIM-2} genes.

Acquired class 1 integrons are considered major contributors of the multi-resistance phenotype expressed by bacteria due the capability of integrons to capture gene cassettes and accommodate them within the variable region of the integron by means of the integrase gene (*int*) and the site specific recombination site (*attI*) and consequently an impressive gene array may result from this fluid capturing machine from the gene pool surrounding the bacteria.

(Bennett, 2008). Gene cassettes include genes encoding functions such as aminoglycoside (Lagatolla *et al.*, 2006; Naas *et al.*, 2006) and trimethoprim modification (Hu *et al.*, 2011). It also comprise major members of the class B β-lactamases family (Jeong *et al.*, 2009; Walsh *et al.*, 2005; Castanheira *et al.*, 2004) and some members of class A and D β-lactamases (Juan *et al.*, 2009).

The detailed characterisation of four Libyan isolates of *P. aeruginosa* indicated the incidence of three different genetic contexts of class 1 integrons. The bla_{VIM-2} positive isolates showed the occurrence of the same integron structures composed of MBL encoding gene bla_{VIM-2} and two aminoglycoside resistance genes; *aadB* and *aac6-II* genes. Similar findings were reported from (Lagatolla *et al.*, 2006; Rojo-Bezares *et al.*, 2011). A novel class 1 integron was detected in *P. aeruginosa* AES83, the integron contained two aminoglycoside resistance genes and one trimethoprim resistance gene. It is composed of the genetic array *aadB1761* and *dfrA1* and *aac6-II*. *P. aeruginosa* isolates AES89 and AES182 showed the incidence of the same integron with exactly the same genetic context; *aadA6* and *ORF* in the variable region of the integrons. The occurrence of this integron has been documented in *P. aeruginosa* by several authors, Naas, *et al.*, reported an In*51* class 1 integron composed of *aadA6* as a novel aminoglycoside adenylyltransferase gene cassettes and an *ORF* which was the first description of the structure of this variable region. (Naas *et al.*, 1999). Similar findings were reported by Shahcheraghi *et al.*, who reported the incidence of 4 integrons with different

gene cassettes arrays acquired by 41 clinical isolates of MDR *P. aeruginosa* in Tehran, Iran, one of the isolates contained a class 1 integron with a variable region of *aadA6* and *ORF* (Shahcheraghi *et al.*, 2010). Some reports described the incidence of this integron as part of complex genetic structure found in *P. aeruginosa* (Nemec *et al.*, 2010), other reports showed the occurrence of this integron as part of complex structure (Naas *et al.*, 2006). It seems that *aadA6* and *ORF* containing integron first discovered in 1999 is the common ancestor and is now found in different geographical areas such France, Iran and now Libya.

Multi-resistant isolates *P. aeruginosa* has been found in different parts of Libyan hospitals, it has been found in ICUs, Chest wards, patients or hospital facilities, and even from the floors of some toilettes in the ICUs. Such emergence of the resistant isolates is a worrisome subject and reveals the lack of a proper hygiene and infection control programs currently operating in Libya. bla_{VIM-2} was identified in one isolate of *P. aeruginosa*, that was collected from stainless steel containers used to keep forceps and other surgical tools in the Chest ward of Aljalla hospital , whereas the other isolate was from a tip of catheter from patient admitted to the ICU of the same hospital in Benghazi.

Chapter Six

Genetic & biochemical characterization of a novel metallo-β-lactamase, TMB-1, from a *Achromobacter xylosoxidans* strain isolated from Tripoli, Libya

6.1 Introduction

The results in this section follow on from the determination of class 1 integrons in *Achromobacter xylosoxidans* (two integrons one at 3kb and one at 2.5kb), one in *Stenotrophomonas maltophilia* (2.5kb) and two isolates of *Citrobacter freundii* each positive for a class integron of 1kb. The isolates were from non-clinical sources from the major hospitals in Tripoli, Libya.

Mobile MBL genes are becoming increasingly frequent and pose a significant challenge to the treatment of Gram-negative infections world-wide such that most MBL-producing organisms are only sensitive to colistin (Cornaglia *et al.*, 2007). These enzymes efficiently hydrolyze all β-lactams, including carbapenems (with the exception of aztreonam), and are located on transferable genetic platforms; namely, either IS*CR* elements or class 1 integrons. The class 1 integrons are sometimes embedded in Tn*21* or Tn*402*-like transposons (Tato *et al.*, 2010). However, several recently characterised MBL genes have been flanked or associated with IS*CR* elements namely, *bla*$_{\text{SPM-1}}$ with IS*CR4*, *bla*$_{\text{NDM-1}}$ with IS*CR1* and *bla*$_{\text{AIM-1}}$ with IS*CR16* (Kumarasamy *et al.*, 2010; Poirel *et al.*, 2004; Toleman *et al.*, 2006).

Several different MBL-type enzymes have been described, among them NDM-1, IMP and VIM derivatives being the most widespread (Bush, 2010). The *bla*$_{\text{IMP-like}}$ (Senda *et al.*, 1996) and *bla*$_{\text{VIM}}$-like (Cornaglia *et al.*, 2000) genes have been identified in clinically relevant bacteria belonging to the Enterobacteriaceae

family, in *Pseudomonas* spp., and in *Acinetobacter* spp. Whilst *bla*$_{NDM-1}$ has mainly been found in Enterobacteriaceae (Bush & Fisher, 2010; Kumarasamy *et al.*, 2010). Several other MBLs have been identified in specific geographical locations, including SIM-1 from *A. baumannii* in Korea (Lee *et al.*, 2005), KHM-1 from *C freundii* in Japan (Sekiguchi *et al.*, 2008), SPM-1 in Brazil (Picao *et al.*, 2009; Toleman *et al.*, 2002), GIM-1 in Germany (Castanheira *et al.*, 2004), and AIM-1 in Australia (Walsh, unpublished data) were all identified in *P. aeruginosa*. As hospitalized patients are subject to infections by Gram-negative bacteria and, in Libya adherence to internationally accepted infection control policies are not optimal, the hospital wards and immediate hospital environment were examined for resistance to extended-spectrum cephalosporins. This study reports these findings and further describes the genetic and biochemical characterization of a novel MBL, TMB-1, from Tripoli, Libya.

6.2 Results

6.2.1 Analysis of samples from Tripoli hospitals

Thirty eight Gram-negative bacteria were able to grow on 10mg/l of ceftazidime (Table D.1 in appendix D). It lists the non-clinical swabs from major hospitals in Tripoli, Libya. All swabs yielded isolates capable of growing on 10mg/l of ceftazidime. The results demonstrate that the environmental isolates collected from the wider Tripoli environment and hospital environment show a high level of resistance to third generation of aminoglycosides and β-lactams. For example, one isolate of *A. xylosoxidans*, AES301, displayed MICs of, 8mg/l, 2mg/l, 4mg/l, 16mg/l, 10mg/l, 32mg/l, and 16mg/l to gentamicin, imipenem, meropenem, cefepime, ceftazidime, cefotaxime, and aztreonam, respectively. Indeed AES301 was sensitive to amikacin and ciprofloxacin (1mg/l) and colistin (0.5mg/l). All isolates grew on media containing ceftazidime and were subsequently screened by the MBL Etest strip to detect the presence of MBL. AES301 gave a positive Etest MBL result and together with the fact it possessed a class 1 integron, was investigated further.

6.2.2 Genetic analysis of carbapenem resistance in *A. xylosoxidans* strain AES301

All isolates were screened for class 1 integrons and mobile genetic elements (Tn*21*, Tn*402*, and IS*CR* elements) and 4 out of 38 isolates were positive for class 1 integrons: one *A. xylosoxidans* (two integrons of one at 3kb and one at

2.5kb), one *S. maltophilia* (2.5kb) and two isolates of *C. freundii* each positive for a class integron of 1kb. None of the isolates were positive for Tn*21*, Tn*402,* and IS*CR* elements. Sequencing analysis of the class 1 integron PCR products from *A. xylosoxidans* AES301, Lasergene package (DNAStar, Madlison, WI) was used to study the nucleotide sequences and the deduced amino acids. The nucleotide sequences were subsequently analysed (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). The two integrons from *A. xylosoxidans* AES301 revealed two near identical integrons; the first possessing the gene cassettes *dhfr*A4-*aac*A4-bla_{OXA-4}, and the second integron-containing the gene cassettes bla_{TMB-1}-*aac*A4-bla_{OXA-4} (Figure 6.1) (Appendix E). The carbapenem resistance could not be mated to either *E. coli* DH5α or *P. aeruginosa* PA01 recipients suggesting the integrons are chromosomally located. This inference was supported by Southern hybridisation data using the bla_{TMB-1}gene as a probe which back-blotted to the *A. xylosoxidans* AES301 chromosome even though it possessed several plasmids.

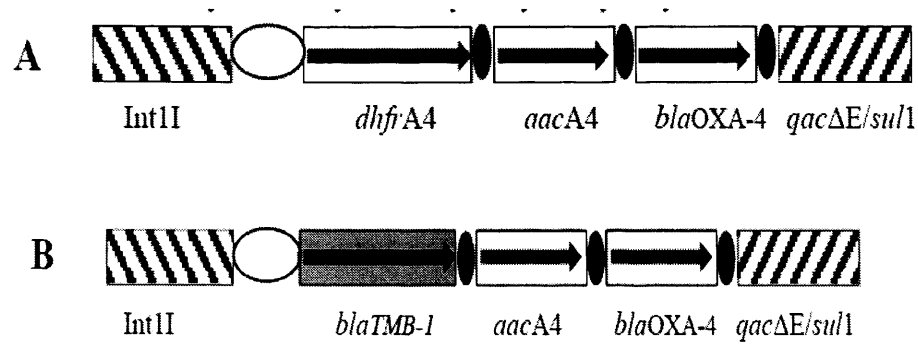

Figure 6.1 Genetic context of two class 1 integrons found in *A. xylosoxidans* AES301 and the primers used to sequence the structures. A. Class 1 integron consisting of the gene cassettes: *dhfr*A4 gene, *aac*A4 gene, *bla*OXA-4 and the *qac*EΔ/*sul*l fusion. B. Class 1 integron consisting of the gene cassettes: *bla*TMB-1, *aac*A4, *bla*OXA-4 and *qac*EΔ/*sul*l genes. The white ellipse represents the hybrid promoter from *IntI*1. The black ellipse represents the 59bp elements at the start of each gene cassette.

6.2.3 Cloning and transconjugation experiments

The results of cloning of the class 1 integron into *E. coli* DH5α produced 3 types of colonies when 50μl of the broth culture was streaked onto L.B. agar plates supplemented with 50 mg/l of Kanamycin, X-galactose and IPTG; white colonies, white colonies with blue spot in the middle and dark blue colonies. Amplification of the class 1 integron from these colonies showed that some white colonies and dark blue colonies produced 2kb and 4kb PCR products which were different from the expected size of class 1 integron used in the cloning experiments. Sequencing of these PCR products did not give

any readable sequences revealing the miss-priming of the oligonucleotides. These results did not produce any cells positive for bla_{TMB-1}. The results of transconjugation experiments showed that the GFP *E. coli* was not able to grow on L.B agar supplemented with 50 mg/l of rifampicin and 4 mg/l of cefotaxime revealing that the bla_{TMB-1} failed to transfer to the GFP *E. coli* and thus is probably chromosomally mediated.

6.2.4 Genomic location of bla_{TMB-1}

The results of PFGE separation of *S*1 digestion of the whole genomic DNA of *A. xylosoxidans* and selected environmental isolates is shown in figure 6.2A. Probing of the PFGE gel of *S*1 digested genomic DNA with radio-labelled bla_{TMB-1} is illustrated in (Figure 6.2B). These results showed that bla_{TMB-1} is located on the chromosome. These results are also in accordance with the cloning and transconjugation experiments that failed to detect any movement of bla_{TMB-1} from parents to recipients predicting the chromosomal location of this bla_{TMB-1}. The results also showed an additional TMB-1 positive isolate of *A. xylosoxidans* which was found in the ICU male surgery ward in Tripoli central hospital.

6.2.5 Comparison of TMB-1 with other MBLs

bla_{TMB-1} contains 735 nucleotides encoding a protein of 245 amino acids and possessing all the key motifs of Ambler class B β-lactamase, SDS gel electrophoresis showed an approximate molecular mass of 25 KDa. At amino acid level, TMB-1 was most closely related to DIM-1 (62%) and GIM-1

(51%), and showed only 48%, 31%, and 29% identity to IMP-1, VIM-2, and NDM-1, respectively (Figure 6.3) (Koh *et al.*, 2004; Poirel *et al.*, 2010; Castanheira *et al.*, 2004; Yong *et al.*, 2009; Garcia-Saez *et al.*, 2008). TMB-1 also possesses virtually the same key residues as DIM-1 that make up the zinc binding residues and the secondary residues supporting the active sites including the putative loop used to facilitating binding of β-lactams during hydrolysis (Figure 6.4). The secondary structural comparison of TMB-1 with VIM-2, (Garcia-Saez *et al.*, 2008) shows that TMB-1 possesses the key zinc binding residues for B1 MBLs; His116, His118, and His196 (zinc 1) and Asp120, Cys221, and His263 (zinc 2) (Figure 6.4).

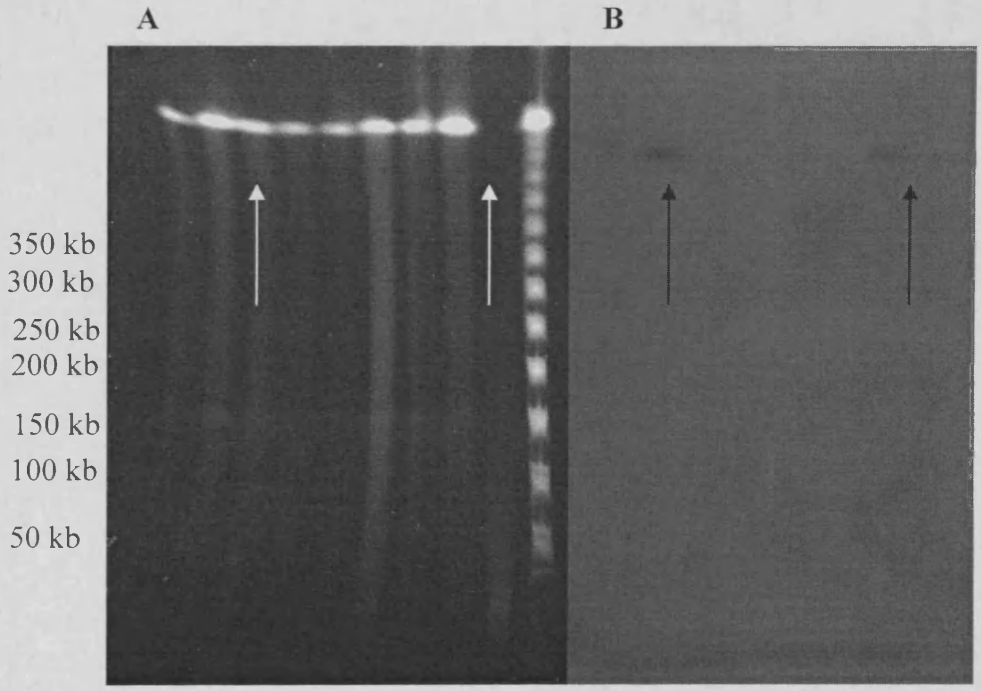

Figure 6.2 Detection of genetic location of bla_{TMB-1} in *A. xylosoxidans*. A. PFGE of *S*1 digested DNA. Lane1: AES301. Lane2: AES302. Lane3: AES303. Lnae4: AES304. Lane5: AES305. Lane6: AES306. Lane7: AES307. Lane8: AES309. Lane9: Marker. B. Autorad after probing with a radio-labelled bla_{TMB-1} of PFGE gel from fig. 6.2A.

The most noticeable difference between TMB-1 and VIM-2 is a gap in the N-terminus of the TMB-1 protein just before the beginning of the first β-sheet (β1, Figure 6.5). This gap in TMB-1 is situated just prior to the "flapping loop" of VIM-2, (Garcia-Saez *et al.*, 2008) further, there are several amino acid differences in this region; namely, (VIM-2 to TMB-1) Q60S, S61R, F62V, D63E, A66G, V67L, and a gap at position 65. This region is also diverse between VIM-2 and VIM-7 where it has been suggested that this contributes to a more flexible "flapping loop" (Borra *et al.*, 2011). Interestingly, DIM-1 possesses the same sequence as TMB-1 in this region with the exception of the gap and the amino acid changes N63E and F65W (DIM-1 to TMB-1) (Poirel *et al.*, 2010). An additional gap in TMB-1 between β7 and β8 compared to VIM-2 is also observed (Garcia-Saez *et al.*, 2008) (Figure. 6.5).

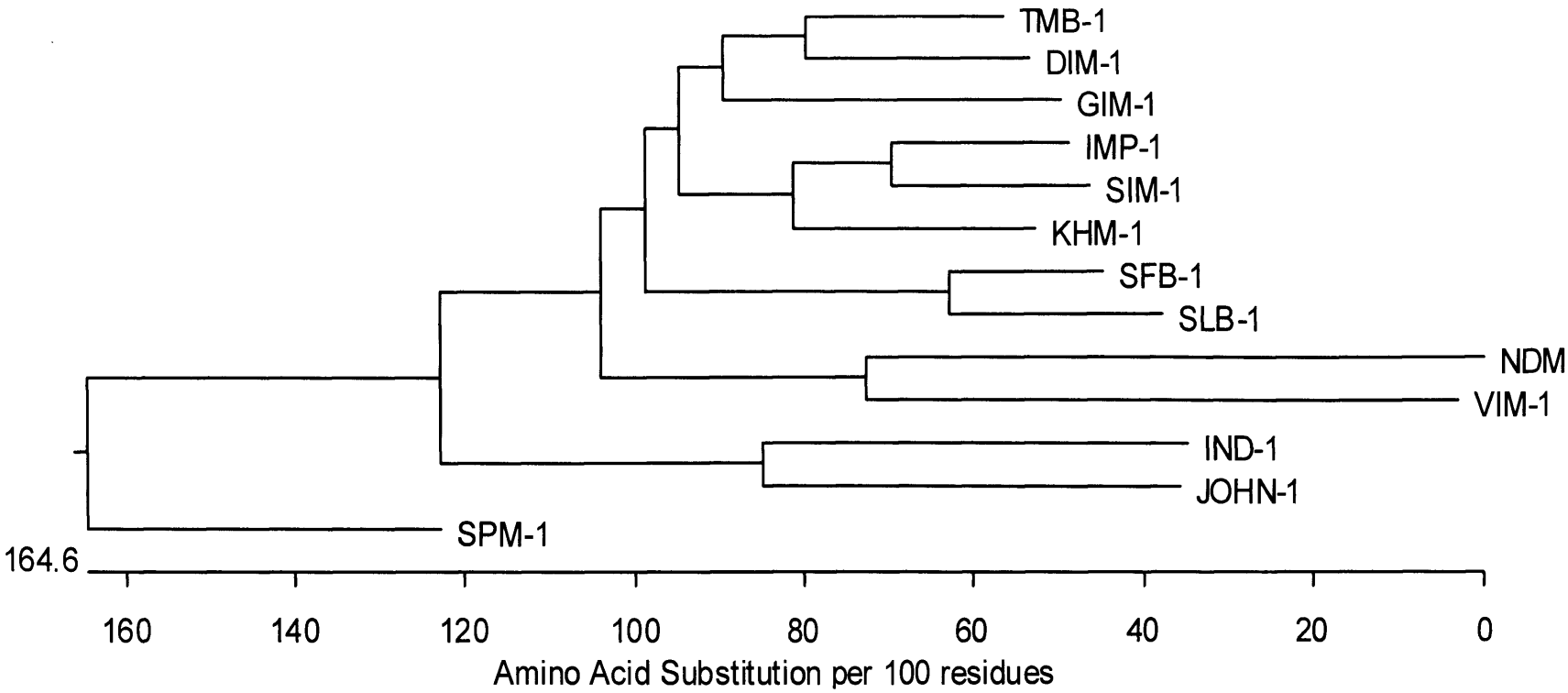

Figure **6.3** Dendrogram of Comparison of amino acid sequence of the β-lactamase TMB-1 and those of other acquired MBLs (DIM-1, GIM-1, IMP-1, KHM-1, NDM-1, VIM-1, SPM-1 and SIM-1) and several naturally occurring MBLs (IND-1 from *Chryseobacterium indologenes*; JOHN-1 from *Flavobacterium johnsoniae*; SLB-1 from *Shewanella livingstonensis*; and SFB-1 from *Shewanella figidimarina*) (Koh *et al.*, 2004; Poirel *et al.*, 2010; Castanaheira *et al.*, 2004; Sekiguchi *et al.*, 2008; Yong *et al.*, 2009; Lee *et al.*, 2005; Toleman *et al.*, 2002; Tato *et al.*, 2010; Naas *et al.*,2003; Poirel *et al.*, 2005; Lin *et al.*, 2005

Figure **6.4** Comparison of amino acid sequence of the β-lactamase TMB-1 and those of other acquired MBLs (DIM-1, GIM-1, IMP-1, KMH-1, NDM-1, VIM-1, SPM-1 and SIM-1) and several naturally occurring MBLs (IND-1 from *Chryseobacterium indologenes*; JOHN-1 from *Flavobacterium johnsoniae*; SLB-1 from *Shewanella livingstonensis*; and SFB-1 from *Shewanella figidimarina*) (Koh *et al*., 2004; Poirel *et al*., 2010; Castanaheira *et al*., 2004; Sekiguchi *et al*., 2008; Yong *et al*., 2009; Lee *et al*., 2005; Toleman *et al*., 2002; Tato *et al*., 2010; Naas *et al*.,2003; Poirel *et al*., 2005; Lin *et al*., 2005). Shaded amino acids are those conserved with TMB-1. β-Lactamase numbering was according to the BBL nomenclature (Galleni *et al*., 2001).

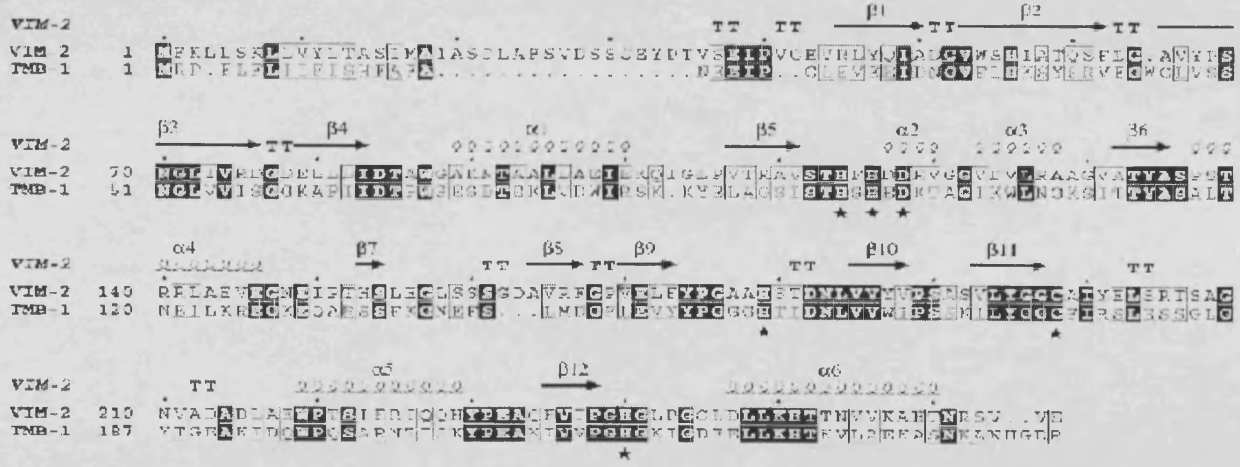

Figure 6.5 Secondary structure of TMB-1 compared to that of VIM-2 (Garcia-Saez *et al.*, 2008). The β- strands and β-helixes are indicated above the TMB-1 sequence. The conserved residues are indicated in black. The conservative amino acid substitutions are boxed. The figure was obtained with ESPript software (http://espript.ibcp.fr/ESPript/ESPript/).

6.2.6 Kinetic properties of TMB-1.

The kinetic properties of TMB-1 were compared with that of DIM-1 and GIM-1 (Table 6.1) and were broadly similar with the exception for the rate of turnover of substrates (*Kcat* values) (Table 6.1). The *Km* values for TMB-1 were similar to DIM-1 and GIM-1 for the penicillins and cephalosporins but were higher for meropenem indicating that meropenem is not a "natural" substrate for TMB-1. The *Kcat* values for TMB-1 were similar for the pencillins compared to GIM-1 but were significantly less (20 to 500-fold) than both DIM-1 and GIM-1 for cefoxitin, cefuroxime and ceftazidime (Poirel *et al.*, 2010; Castanheira *et al.*, 2004) (Table 6.1). TMB-1 also possessed lower *Kcat* values for the carbapenems (3 to 30-fold) compared to DIM-1 and GIM-1. These data further showed that the efficiency of the enzyme (*Kcat/Km*) was significantly lower for the cephalosporins and carbapenems (Table 6.2). Such differences in kinetic values is interesting given that TMB-1 and DIM-1 are similar and that their sequence over the "VIM-2 flapping loop" is nearly identical, further suggesting that the reasons for these kinetic differences could lie elsewhere in the TMB-1 structure (Figure 6.5).

Table 6.1 Steady-state kinetic constants of TMB-1, DIM-1 and GIM-1 (Poirel *et al.*, 2010; Castanheira *et al.*, 2004)

Compound	TMB-1			DIM-1 [a]			GIM-1 [b]		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} / K_m (s^{-1}/μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} / K_m (s^{-1}/μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} / K_m (s^{-1}/μM)
Ampicillin	3.3	27	0.122	20	110	0.182	3.3	20	0.165
Piperacillin	3.3	72	0.046	NR	NR	NR	6.9	69	0.1
Cefoxitin	0.3	69	0.004	8	20	0.4	8.3	206	0.04
Cefuroxime	0.1	9	0.011	NR	NR	NR	5.9	7	0.843
Ceftazidime	0.07	31	0.002	3	50	0.06	18	31	0.58
Ertapenem	0.4	31	0.013	NR	NR	NR	NR	NR	NR
Imipenem	1.7	200	0.009	35	80	0.438	27	287	0.094
Meropenem	1.4	75	0.019	50	10	5	2.7	25	0.108
Aztreonam	<0.01	ND	ND	<0.01	ND	ND	ND	ND	ND

[a]Poirel *et al.*, 2010

[b]Castanheira *et al.*,2004

cNR: Not Reported

dND: Not Detected

6.3 Discussion

Non-clinical isolates collected from the major Tripoli hospitals were able to grow on media containing ceftazidime. MDR Gram-negative bacteria e.g *A. xylosoxidans*, *P. aeruginosa*, *A. baumannii*, *E. cloacae* and *C. freundii* were detected in the hospital environment inside and around the hospitals. The occurrence of MDR strains in the clinical setting of Tripoli hospitals reveals the lack of hospital hygiene in these hospitals.

Investigation into the incidence of antibiotic resistance genes embedded in class 1 integrons was surprising. Two class 1 integrons were detected in *A. xylosoxidans*, the 3kb integron had a novel MBL gene (bla_{TMB-1}) in the first position followed by two antibiotic resistance genes *aac*A4 and bla_{OXA-4}, whereas the 2kb composed of *dhfrA4*, *aac*A4 and bla_{OXA-4}. The occurrence of such integrons is unusual in terms of their genetic context and more importantly the discovery of a novel MBL in a non-clinical isolate because of the rarity of environmental MBLs whose genes are shown to be mobile.

The occurrence of *A. xylosoxidans* in the non-clinical settings of Tripoli hospitals may perhaps indicate the dissemination of bla_{TMB-1} in Tripoli and across Libya. The occurrence of bla_{TMB-1} in an environmental strain may raise the question of whether MBLs are originated from environmental bacteria as

the case of origin of $bla_{CTX\text{-}M\text{-}3}$ form *Kluyvera ascorbata* proposed by (Rodriquez *et al.*, 2004).

TMB-1 has all the key motifs of Ambler class B β-lactamases; it shares 62% similarity with DIM-1 (Poirel *et al.*, 2010) and 51% with GIM-1 (Castanheira *et al.*, 2004). TMB-1 and DIM share the same key residues that facilitate binding of these enzymes to β-lactam antibiotics during hydrolysis. Secondary structure of TMB-1 showed that these enzymes posses all the key zinc binding residues (His116, His118 and His196) required for zinc1 activity and (Asp120 and His263 for zinc 2 activities) as reported for all class B1 MBLs (Osano *et al.*, 1994; Lauretti *et al.*, 1999; Toleman *et al.*, 2002; Castanheira *et al.*, 2004; Lee *et al.*, 2005; Gupta, 2008; Sekiguchi *et al.*, 2008; Yong *et al.*, 2009; Poirel *et al.*, 2010).

Achromobacter is not a key pathogen although a growing number reports indicate that it is capable of causing UTIs (Tena *et al.*, 2008), ocular infections (Reddy *et al.*, 2009), contamination of dialysis (Turgutalp *et al.*, 2011) and ultrasound equipment (Olshtain-Pops *et al.*, 2011) and can cause additional complications in cystic fibrosis patients (Lambiase *et al.*, 2011; Ridderberg *et al.*, 2011). Interestingly, although AES301 carrying TMB-1 was found from a ward surface swab, the same strain could not be identified from a clinical source although in Libya clinical diagnostic microbiology may not normally scrutinize strains to species level. To date only two cases of MBL genes (both

bla$_{VIM-2}$) have been reported from *Achromobacter* spp. – from Greece (Sofianou *et al.*, 2005) and Korea (Shin *et al.*, 2005) and both carried in class 1 integrons. All other MBLs discovered IMP; VIM; SPM-1; GIM-1; SIM-1; AIM; KHM-1; NDM-1 and DIM-1 were detected in clinical isolates from patients suffered from serious infections. (Osano *et al.*, 1994; Lauretti *et al.*, 1999; Toleman *et al.*, 2002; Castanheira *et al.*, 2004; Lee *et al.*, 2005; Gupta, 2008; Sekiguchi *et al.*, 2008; Yong *et al.*, 2009; Poirel *et al.*, 2010).

This is the first MBL reported from Libya and being a new MBL subclass B1 provides further evidence of the structural heterogeneity of this group of β-lactamases.

Chapter Seven
General Discussion

This study investigated the mechanism of antibiotic resistance in randomly collected isolates of Gram-negative bacteria in Tripoli and Benghazi (Figure 7.1). The isolates were from clinical samples recovered from patients admitted to the hospitals and from the hospital environment and for the purpose of my thesis these are regarded as non-clinical samples. These swabs were from floors, walls, bedsides, toilets, workstation tables, mechanical ventilators, oxygen suppliers, stainless steel containers, curtains, baby incubators, trolleys and other instruments used in the hospitals in particular ICUs. The non-hospital environmental isolates were swabs collected from streets in Tripoli and Benghazi; the samples were from floors and dusty areas in the streets.

Figure 7.1 Map of Libya showing the important cities and their locations (http://www.google.co.uk/imgres?q=libyan+map&hl=en&gbv=2&tbm=isch

The data in my thesis clearly demonstrates the emergence of MDR Gram-negative bacteria in hospital settings that include clinical and non-clinical isolates. In total, 171 isolates were recovered for this study.

Enterobacteriaceae represent the most numerous of the Gram-negative with *K. pneumoniae* as the most frequently isolated bacteria. High prevalence of ESBLs was detected among *K. pneumoniae* and *E. coli*, more importantly $bla_{\text{CTX-M}}$ group1 were found widely disseminated in association with IS*Ecp1* being located immediately upstream of most of the ESBL encoding gene.

The highest incidence of $bla_{\text{CTX-M}}$ group1 was detected among clinical and non-clinical isolates collected from hospitals. *K. pneumoniae* and *E. coli* isolates tested for the occurrence of conjugative plasmids responsible for the movement of $bla_{\text{CTX-M}}$ group1/IS*Ecp1*. The study showed that these plasmids can move $bla_{\text{CTX-M-15}}$/IS*Ecp1* genes from the resistant isolates to sensitive *E. coli*, leading to *E. coli* transconjugants expressing an MDR phenotype. It is worth mentioning that plasmid mediated $bla_{\text{CTX-M-15}}$ associated with IS*Ecp1* has been detected in an environmental isolate of *K. pneumoniae* AES817 (ST511) cultured from one of Benghazi streets – this isolate clearly has not been recently exposed to antibiotics. These findings are an alarmingly indication that an outbreak of MDR *K. pneumoniae* and *E. coli* isolates can occur in different hospitals that provide different services to patients; these hospitals are maternity hospitals, pediatric hospitals, surgical hospitals and general hospital in Tripoli and Benghazi.

The occurrence of *K. pneumoniae* and *E. coli* in Libyan hospitals is interesting in terms of the prevalence of clonally and non-clonally related $bla_{\text{CTX-M-15}}$

positive isolates (Lee *et al.*, 2011; Webster *et al.*, 2011; Alfaresi *et al.*, 2011; Fam *et al.*, 2011; Al Sweih *et al.*, 2011). The collection of isolates being clinical, non-clinical or environmental was non-representative seeking the occurrence of any resistance mechanism in these isolates. Among all the isolates positive for $bla_{\text{CTX-M}}$ group1, 4 pairs of *K. pneumoniae* isolates were found clonally related. One pair represented two isolates that were found in two different hospitals in Benghazi - isolates AES135 and AES140 that were cultured from urine and blood samples, respectively.

The occurrence of several clonally related *K. pneumoniae* and *E. coli* that resulted from the non-representative sample collection reflected the total lack of appropriate infection control programs in Libyan hospitals. Lack of hospital hygiene is another reason that has contributed to the emergence and spread of multi-drug resistant isolates of *K. pneumoniae* and *E. coli* in Libyan hospitals. The overuse of extended- spectrum cephalosporins to treat infections in Libya may be another significant factor for the appearance of clonally and non-clonally related isolates of *K. pneumoniae* and *E. coli* in Libya. (Ito & Kamimura, 2011; Wang *et al.*, 2011)

The occurrence of $bla_{\text{CTX-M}}$ group1/IS*Ecp1*on different plasmid sizes in *K. pneumoniae* and *E. coli* isolates suggest the movement of these genes by conjugative plasmids (Lavollay, *et al.*, 2006) as shown from data on mating studies in a subset of these isolates. The ability of these isolates to mobilize

and facilitate the spread of antibiotic resistance genes may explain the frequency of $bla_{CTX\text{-}M}$ group1/IS*Ecp1* in non-clonally related species of *K. pneumoniae* and *E. coli* (Woodford *et al.*, 2004; Gonullu *et al.*, 2008; Lavollay *et al.*, 2006, Yu & K. Cheng, 2004; Ramdani-Bouguessa, *et al.*, 2006; Abbassi et *al.*, 2008). A possible explanation for the different plasmid location of $bla_{CTX\text{-}M}$ group1 is due to the presence of the insertion sequence, IS*Ecp1* that is known to move and promote the expression of the ESBL gene. Two suggested mechanisms of $bla_{CTX\text{-}M}$ group1 acquisition are proposed: the movement of $bla_{CTX\text{-}M}$ group1 by conjugative plasmids and the role of the insertion sequence IS*Ecp1* in mobilizing $bla_{CTX\text{-}M}$ group1 within the strains (Abbassi et *al.*, 2008; Poirel *et al.*, 2003; Naseer & Sundsfjord, 2011; Younes *et al.*, 2011; Gonullu *et al.*, 2008; Villa *et al.*, 2010; Partridge *et al.*, 2011).

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was identified in this study as ST147 which is known as a world wide ST contributing to the emergence of $bla_{CTX\text{-}M\text{-}15}$ genes. The genetic context of this integron has been reported in clinical isolates of *E. coli* in Asia and Europe (Yu *et al.*, 2004; Tang *et al.*, 2001; Saenz *et al.*, 2009; Vinue *et al.*, 2008)

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The *Km* values for TMB-1 are similar to DIM-1 and GIM-1 for penicillins and cephalosporins but are larger for meropenem indicating that meropenem is not a "natural" substrate for TMB-1. Rather like many other MBLs, the origin of TMB-1 will never be known. Any MBL should be regarded important even if the kinetics are less impressive than previously reported MBLs, particularly those encoded by mobile genes via IS*CR*s, transposons, ICEs or integrons carried on plasmids. For example, bla_{NDM-1} has appeared in *K. pneumoniae*,

E. coli and other Enterobacteriaceae in the clinical setting as well as the environment outside the hospitals and carried on a variety of different plasmids with the immediate environment surrounding bla_{NDM-1} highly variable (Walsh *et* al., 2011; Poirel *et al.*, 2011). It is the same scenario for the dissemination of MBL encoding genes in environmental strains that share very large genetic pool with numerous strains of bacteria possibly leading to the emergence of MBLs in clinical strains originating from the environment.

The findings of this study show the presence of several antibiotic resistance mechanisms among Enterobacteriaceae and non-fermenters in Libya as shown by the incidence of clonally and non-clonally related *K. pneumoniae* and *E. coli* carrying $bla_{CTX-M-15}$/IS*Ecp1*, the dissemination of class 1 integrons that carry MBL genes and other β-lactamase genes as well as aminoglycoside and trimethoprim resistance genes. This study, moreover, tried to associate the emergence of ESBLs and other resistance mechanisms with clonality to further our understanding on how the transmission of antimicrobial resistance in Libyan hospitals occurs. The study also determined the MBLs, VIM-2 and TMB-1, as responsible for high-level β-lactam resistance in non-fermenters, *P. aeruginosa* and *A. xylosoxidans* shown in the clinical and non-clinical settings and health care facilities in Tripoli and Benghazi.

It is worth mentioning that acute care facilities are among the most common sites for the development of antimicrobial resistance. The intensive uses of

antimicrobial agents as well as suboptimal infection control policies are major factors affecting the emergence and transmission of antibiotic resistance bacteria in Libyan hospitals. Antibiotic resistance genes can be used as a genetic marker for bacterial outbreaks and help in the early detection of outbreaks bacterial infections and thus might effectively reduce the cost of managing outbreaks, decrease morbidity and mortality by eliminating the nosocomial pathogens and consequently enhance the application of infection control programs. In contrast Libya has few of these capabilities even before the civil war and the data produced in this thesis are the first studies of this kind to be undertaken. Prior to my research, there were no reports of resistance genes, mobile elements. Libyan hospitals lack a systematic approach to patient management – for example, hospitals in Benghazi can get ampicillin/sulbactum but hospitals in Tripoli could not. Microbiology laboratories were also desperately inadequate with no standardization of media or susceptibility testing. Infection control programs were also absent with no instructions to clinical staff on decreasing infections and, hand-washes and disposal towels are completely absent.

Thus, it is hoped that the data from this thesis together with a new political beginning for Libya can help build an awareness of resistance and how the monitoring of resistance genes and mobile genetic elements can aid and enhance patient outcome. The potential to improve the laboratory and clinical infra-structure in Libya is enormous and, with an awareness of operating

systems in laboratories in other countries, together with a desire to be more transparent in implementing them, patient outcome will hopefully be dramatically improved.

I trust and hope the data from my thesis is merely the beginning.

Chapter Seven

General Discussion

This study investigated the mechanism of antibiotic resistance in randomly collected isolates of Gram-negative bacteria in Tripoli and Benghazi (Figure 7.1). The isolates were from clinical samples recovered from patients admitted to the hospitals and from the hospital environment and for the purpose of my thesis these are regarded as non-clinical samples. These swabs were from floors, walls, bedsides, toilets, workstation tables, mechanical ventilators, oxygen suppliers, stainless steel containers, curtains, baby incubators, trolleys and other instruments used in the hospitals in particular ICUs. The non-hospital environmental isolates were swabs collected from streets in Tripoli and Benghazi; the samples were from floors and dusty areas in the streets.

Figure 7.1 Map of Libya showing the important cities and their locations (http://www.google.co.uk/imgres?q=libyan+map&hl=en&gbv=2&tbm=isch

The data in my thesis clearly demonstrates the emergence of MDR Gram-negative bacteria in hospital settings that include clinical and non-clinical isolates. In total, 171 isolates were recovered for this study.

Enterobacteriaceae represent the most numerous of the Gram-negative with *K. pneumoniae* as the most frequently isolated bacteria. High prevalence of ESBLs was detected among *K. pneumoniae* and *E. coli*, more importantly $bla_{CTX\text{-}M}$ group1 were found widely disseminated in association with IS*Ecp1* being located immediately upstream of most of the ESBL encoding gene.

The highest incidence of $bla_{CTX\text{-}M}$ group1 was detected among clinical and non-clinical isolates collected from hospitals. *K. pneumoniae* and *E. coli* isolates tested for the occurrence of conjugative plasmids responsible for the movement of $bla_{CTX\text{-}M}$ group1/IS*Ecp1*. The study showed that these plasmids can move $bla_{CTX\text{-}M\text{-}15}$/IS*Ecp1* genes from the resistant isolates to sensitive *E. coli*, leading to *E. coli* transconjugants expressing an MDR phenotype. It is worth mentioning that plasmid mediated $bla_{CTX\text{-}M\text{-}15}$ associated with IS*Ecp1* has been detected in an environmental isolate of *K. pneumoniae* AES817 (ST511) cultured from one of Benghazi streets – this isolate clearly has not been recently exposed to antibiotics. These findings are an alarmingly indication that an outbreak of MDR *K. pneumoniae* and *E. coli* isolates can occur in different hospitals that provide different services to patients; these hospitals are maternity hospitals, pediatric hospitals, surgical hospitals and general hospital in Tripoli and Benghazi.

The occurrence of *K. pneumoniae* and *E. coli* in Libyan hospitals is interesting in terms of the prevalence of clonally and non-clonally related $bla_{CTX\text{-}M\text{-}15}$

positive isolates (Lee *et al.*, 2011; Webster *et al.*, 2011; Alfaresi *et al.*, 2011; Fam *et al.*, 2011; Al Sweih *et al.*, 2011). The collection of isolates being clinical, non-clinical or environmental was non-representative seeking the occurrence of any resistance mechanism in these isolates. Among all the isolates positive for $bla_{\text{CTX-M}}$ group1, 4 pairs of *K. pneumoniae* isolates were found clonally related. One pair represented two isolates that were found in two different hospitals in Benghazi - isolates AES135 and AES140 that were cultured from urine and blood samples, respectively.

The occurrence of several clonally related *K. pneumoniae* and *E. coli* that resulted from the non-representative sample collection reflected the total lack of appropriate infection control programs in Libyan hospitals. Lack of hospital hygiene is another reason that has contributed to the emergence and spread of multi-drug resistant isolates of *K. pneumoniae* and *E. coli* in Libyan hospitals. The overuse of extended- spectrum cephalosporins to treat infections in Libya may be another significant factor for the appearance of clonally and non-clonally related isolates of *K. pneumoniae* and *E. coli* in Libya. (Ito & Kamimura, 2011; Wang *et al.*, 2011)

The occurrence of $bla_{\text{CTX-M}}$ group1/IS*Ecp1*on different plasmid sizes in *K. pneumoniae* and *E. coli* isolates suggest the movement of these genes by conjugative plasmids (Lavollay, *et al.*, 2006) as shown from data on mating studies in a subset of these isolates. The ability of these isolates to mobilize

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Chapter Eight

Appendices

Appendix A

Table A.1 Antibiotics and chemicals used in selection of antibiotic resistant strains experiments

Antibiotic	Source
Ceftazidime	SIGMA-ALDRICH
Rifampicin	SIGMA-ALDRICH
Meropenem	SIGMA-ALDRICH
Ertapenem	SIGMA-ALDRICH
Imipenem	SIGMA-ALDRICH
Cefoxitin	SIGMA-ALDRICH
Cefuroxone	SIGMA-ALDRICH
Ampicillin	SIGMA-ALDRICH
Piperacillin	SIGMA-ALDRICH
Sodium azide	SIGMA-ALDRICH
Kanamycin	SIGMA-ALDRICH

Appendix A

Table A.2 Oligonucleotides used for PCR amplification and DNA sequencing

Gene target	Primer name	Primer sequence	Reference
*bla*CTX-M15	CTX-M-15 F	5'GTTCACGCTGATGGCGACGGC'3	This study
*bla*CTX-M-15	CTX-M-15 R	5'GACGCTAATACATCGCGACGGC'3	This study
Beginning of *ISEcp1*	ISEcpu2	5' AATACTACCTTGCTTTCTGA '3	Leflon-Guibout *et al.*, 2004
The end of IS*Ecp1*	ISEcpu1	5'AAAAATGATTGAAAGGTGGT'3	Ho *et al.*, 2005
Integrase gene	VAF	5'GCCTGTTCGGTTCGTAAGCT'3	Levesque *et al.*, 1994
Transposase gene	tniC	5'CGATCTCTGCGAAGAACTCG'3	Toleman *et al.*, 2007
Quaternary Ammonium Compound	QacR	5'CGGATGTTGCGATTACTTCG'3	Levesque, C. *et al.*, 1994
Transposon	TniC	5'CGATCTCTGCGAAGAACTCG'3	Mammeri, H. *et al.*, 2003
*bla*TEM	TEM F	5'TCCGCTCATGAGACAATAACC'3	Kiratisin *et al.*, 2008
*bla*TEM	TEM R	5'TTGGTCTGACAGTTACCAATGC'3	Kiratisin *et al.*, 2008
*bla*SHV	SHV F	5'TGGTTATGCGTTATATTCGCC'3	Kiratisin *et al.*, 2008
*bla*SHV	SHV R	5'GGTTAGCGTTGCCAGTGCT'3	Kiratisin *et al.*, 2008
*ISCR*1	ISCR1 F	5'GGT TGC AAC GAC TCA AGCG'3	Lee, K. *et al.*, 2005
*ISCR*1	ISCR1 R	5'CAC TCG TTT ACC GCT CAA GC'3	Lee, K. *et al.*, 2005
blaampC MOX	MOX F	5'GCT GCT CAA GGA GCA CAG GAT'3	Brolund *et al.*, 2010
blaampC MOX	MOX R	5'CAC ATT GAC ATA GGT GTG GTG C'3	Brolund *et al.*, 2010
blaampC CITM	CITM F	5'TGG CCA GAA CTG ACA GGC AAA '3	Brolund *et al.*, 2010
blaampC CITM	CITM R	5'TTT CTC CTG AAC GTG GCT GGC'3	Brolund *et al.*, 2010
blaampC DHAM	DHAM F	5'AAC TTT CAC AGG TGT GCT GGGT'3	Brolund *et al.*, 2010
blaampC DHAM	DHAM R	5'CCG TAC GCA TAC TGG CTT TGC'3	Brolund *et al.*, 2010
blaampC ACCM	ACCM F	5'AAC AGC CTC AGC AGC CGG TTA'3	Brolund *et al.*, 2010
blaampC ACCM	ACCM R	5'TTC GCC GCA ATC ATC CCT AGC'3	Brolund *et al.*, 2010
blaampC EBCM	EBCM F	5'TCG GTA AAG CCG ATG TTG CGG '3	Brolund *et al.*, 2010
blaampC EBCM	EBCM R	5'CTT CCA CTG CGG CTG CCA GTT'3	Brolund *et al.*, 2010
blaampC FOXM	FOXM F	5'AAC ATG GGG TAT CAG GGA GAT G'3	Brolund *et al.*, 2010
blaampC FOXM	FOXM R	5'CAA AGC GCG TAA CCG GAT TGG'3	Brolund *et al.*, 2010

Table A.3. Oligonucleotides used for PCR amplification and DNA sequencing

Gene target	Primer name	Primer sequence	Reference
TMB-1	Trip-1F61	5'GCC AAC GAA GAA ATA CCC GC'3	This study
TMB-1	Trip2-10	5'TGG GCT AGG TTA CAC TGG TG'3	This study
TMB-1	Trip617R	5'TTC TAG CGG ATT GTG GCC AC'3	This study
TMB-1	Trip2	5'CAA GGA GCT CAT TCA AAGG'3	This study
TMB-1	Trip1	5'GGA GCA GGC AAG GAG CT'3	This study
TMB-1	Trip4	5'AAG GGT TAA CAA GTG GCA GC'3	This study
TMB-1	Trip75	5'ACC CGG ATT GGA AGT TGA GG'3	This study
TMB-1	Trip1 FF	5'TGA TCA GTG GCC ACA ATC CG'3	This study
TMB-1	Trip1 F	5'CGG ATT GTG GCC ACT GAT CA'3	This study
TMB-1	Trip3	5'GGC CAT ACT AAT GAT AAC'3	This study
dhfrA	dhfrA 1FR	5'CCC GAT AAC TCC ATT CTT CG'3	This study
dhfrA	dhfrA 1F	5'CGA AGA ATG GAG TTA TCG GG'3	This study
dhfrA	dhfrA 1R	5'GTT AGA GGC GAA GTC TTG GG'3	This study
dhfrA	dhfrA 1FF	5'CCC AAG ACT TCG CCT CTA AC'3	This study
aac6II	aac6II R	5'GGC GTC GGC TTG AAT GAG TT'3	This study
aac6II	aac6II F	5'AAG TGG CAG CAA CGG ATT CG'3	This study
aac6II	aac6II FR	5'GAA TCC GTT GCT GCC ACT TG'3	This study
aac6II	aac6II FF	5'CAA CTC ATT CAA GCC GAC GC'3	This study
aac6II	aac6II FR	5'GTG CTC GCG GAC ATG AAA TG'3	This study
Oxa-4	Oxa-4-FR	5'CAC TTA TGG CAT TTG ATG CG'3	This study
Oxa-4	Oxa-4-F	5'CGC ATC AAA TGC CAT AAG TG'3	This study
SPM-1D F	*bla*SPM-1	5'CCT ACA ATC TAA CGG CGA CC'3	Zavascki *et al.*, 2005
SDPM-1D R	*bla*SPM-1	5'TCG CCG TGT CCA GGT ATA AC'3	Zavascki *et al.*, 2005
IMP-2 F	*bla*IMP-2	5'GGC AGT CGC CCT AAA ACA AA'3	Wu *et al.*, 2007
IMP2 R	*bla*IMP-2	5'TAG TTA CTT GGC TGT GAT GG'3	Wu *et al.*, 2007
IMP-1 R	*bla*IMP-1	5'TTA GTT GCT TGG TTT TGA TG'3	Queenan & Bush, 2007
IMP-1 F	*bla*IMP-1	5'TGA GCA AGT TAT CTG TAT TC'3	Queenan & Bush, 2007
VIM- F	*bla*VIM	5'GTC TAT TTG ACC GCG TC'3	Cezario *et al.*, 2009
VIM- R	*bla*VIM	5'CTA CTC AAC GAC TGA GCG'3	Cezario *et al.*, 2009
GIM- F	*bla*GIM-1	5'AGA ACC TTG ACC GAA CGC AG'3	Queenan & Bush, 2007
GIM-R	*bla*GIM-1	5'ACTCATGACTCCTCACGAGG'3	Queenan & Bush, 2007
NDM F	*bla*NDM-1	5'GAA GCT GAG CAC CGC ATTA G'3	Sidjabat *et al.*, 2010
NDM R	*bla*NDM-1	5'TGC GGG CCG TAT GAG TGA TT'3	Sidjabat *et al.*, 2010
DIM-1 F	*bla*DIM-1	5' GCT TGT CTT CGC TTG CTA ACG '3	Poirel *et al.*, 2011
DIM-1 R	*bla*DIM-1	5' CGT TCG GCT GGA TTG ATT TG '3	Poirel *et al.*, 2011
SIM-1 F	*bla*SIM-1	5' TAC AAG GGA TTC GGC ATC G '3	Poirel *et al.*, 2011
SIM-1 R	*bla*SIM-1	5' TAA TGG CCT GTT CCC ATG TG '3	Poirel *et al.*, 2011
KHM-1 F	*bla*KHM-1	5' GGT ATG CGC TGA CGA TTC '3	Sekiguchi *et al.*, 2008
KHM-1 R	*bla*KHM-1	5' TTT ATT TGG TGG CTG TTT TGT C '3	Sekiguchi *et al.*, 2008
AIM-1 F	*bla*AIM-1	5' CTG AAG GTG TAC GGA AAC AC '3	Poirel *et al.*, 2011
AIM-1 R	*bla*SIM-1	5' GTT CGG CCA CCT CGA ATT G '3	Poirel *et al.*, 2011

Table A.4. Primers, target site and size of replicons tested

Plasmid	DNA sequence	Target site	Gene size (bp)
Multiplex 1			
HI1 FW	5'-gga gcg atg gat tac ttc agt ac 3	parA-parB	471
HI1 RV	5'-tgc cgt ttc acc tcg tga gta 3		
HI2 FW	5'-tttc tcc tga gtc acc tgt taa cac 3	Iterons	644
HI2 RV	5'-ggc tcac tac cgt tgt cat cct 3		
I1 FW	5'-cga aag ccg gac ggc agaa 3	RNA1	139
I1 RV	5'-tcg tcgt tcc gcc aag ttc gt 3		
Multiplex 2			
X FW	5'-aac ctt aga ggc tat tta agt tgc tgat '3	ori γ	376
X RV	5'-tga gag tca att ttt atc tca tgt ttt agc '3		
L/M FW	5'-gga tga aaa cta tca gca tct gaa g '3	repA,B,C	785
L/M RV	5'-ctg cag ggg cga ttc ttt agg '3		
N FW	5V-gtc taa cga gct tac cga ag '3	repA	559
N RV	5'-gtt tca act ctg cca agt tc '3		
Multiplex 3			
FIA FW	5'-cca tgct ggt tct aga gaa ggtg '3	Iterons	462
FIA RV	5'-gta tat cct tac tgg ctt ccg cag '3		
FIB FW	5'-gga gtt ctg aca cac gat ttt ctg '3	repA	702
FIB RV	5'-ctc ccg tcg ctt cag ggc att '3		
W FW	5'-cct aag aac aac aaa gcc cccg '3	repA	242
W RV	5'-ggt gcg cgg cat aga acc gt '3		
Multiplex 4			
Y FW	5'-aat tca aac aac act gtg cag cctg '3	repA	765
Y RV	5'-gcg aga atg gac gat tac aaa act tt '3		
P FW	5'-cta tgg ccc tgc aaa cgc gcc aga aa '3	Iterons	534
P RV	5'-tca cgc gcc agg gcg cag cc '3		
FIC FW	5'-gtg aac tgg cag atg agg aagg '3	repA2	262
FIC RV	5'-ttc tcc tcg tcg cca aac tag at '3		
Multiplex 5			
A/C FW	5'-gag aac caa aga caa aga cct gga 3'	repA	465
A/C RV	5'-acg aca aac ctg aat tgc ctc ctt '3		
T FW	5'-ttg gcc tgt ttg tgc cta aac cat '3	repA	750
T RV	5'-cgt tga tta cac tta gct ttg gac '3		
FIIs FW	5'-ctg tcg taa gct gat ggc '3	repA	270
FIIs RV	5'-ctc tgc cac aaa ctt cagc'3		
Simplex 1			
F^{rep} FW	5'-tga tcg ttt aag gaa ttt tg '3	RNA1/repA	270
F^{rep} RV	5'-gaa gat cag tca cac cat cc '3		
Simplexs 2 and 3			
K/B FW	5'-gcg gtc cgg aaa gcc aga aaac '3	RNA1	160
K/B RV	5'-tct ttc acg agc ccg cca aa '3		
B/O RV	5'-tct gcg ttc cgc caa gtt cga '3	RNA1	159

Appendix B

Figures of multiplex PCR to detect the occurrence of CTX-M type ESBLs in *K. pneumoniae* (Figures B.1-B.5 in the next three pages)

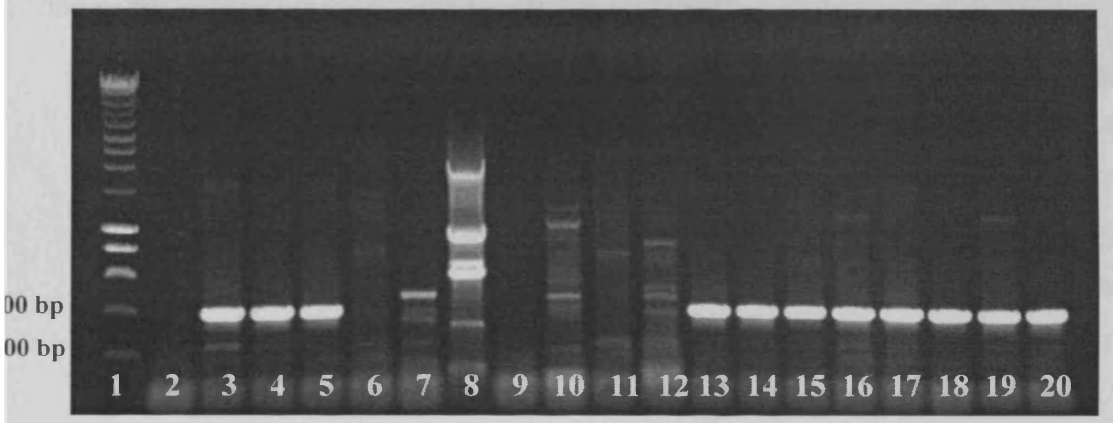

Figure B.1 Multiplex PCR experiment to detect the incidence of CTX-M type ESBLs groups 1,2,8,9 and 26 in *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES152. Lane3: AES170. Lane4: AES172. Lane5: AES178. Lane6: AES117. Lane7: AES197. Lane8: AES187. Lane9: AES188. Lane10: AES194. Lane11: AES 203. Lane12: AES216. Lane13: AES225. Lane14: AES236. Lane15: AES258. Lane16: AES260. Lane17: AES261. Lane18: AES265. Lane19: AES268. Lane20: AES270.

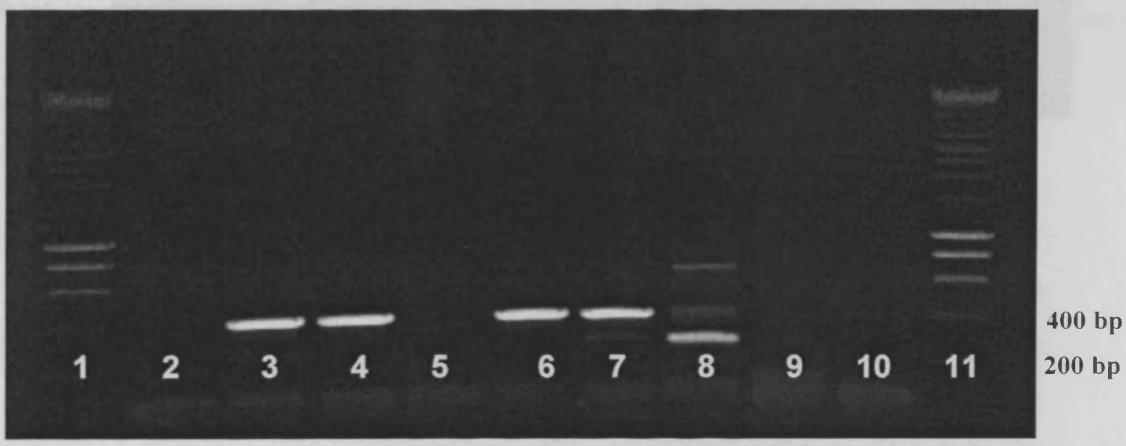

Figure B.2 Multiplex PCR experiment to detect the incidence of CTX-M type ESBLs groups 1,2,8,9 and 26 in *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES271. Lane3: AE273. Lane4: AES274. Lane5: AES275. Lane6: AES279. Lane7: AES280. Lane8: AES917. Lane9: AES942. Lane10: H2O. Lane11: Marker.

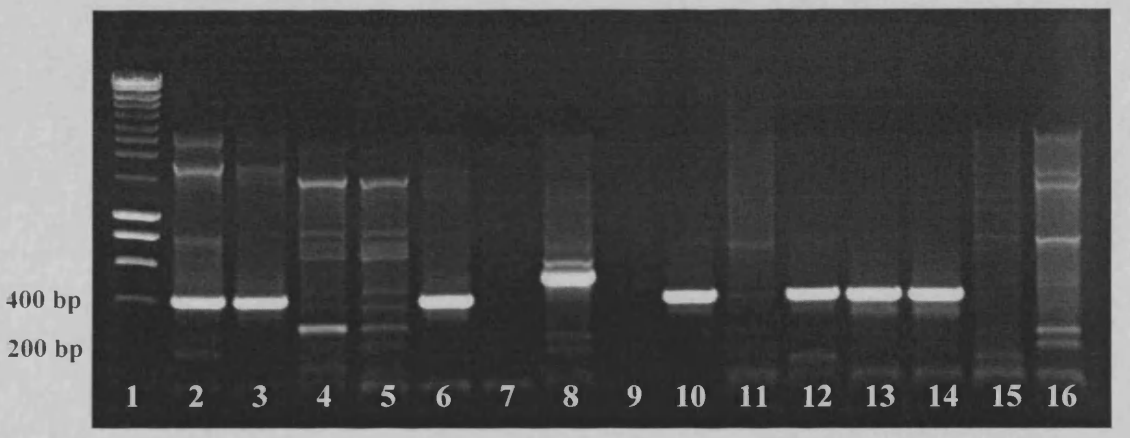

Figure B.3 Multiplex PCR experiment to detect the incidence of CTX-M type ESBLs groups 1,2,8,9 and 26 in *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES506. Lane3: AES722. Lane4: AES808. Lane5: AES809. Lane6: AES817. Lane7: AES836. Lane8: AES936. Lane9: AES939. Lane10: AES943. Lane11: AES960. Lane12: AES961. Lane13: AES970. Lane14: AES973. Lane15: AES975. Lane16: AES977.

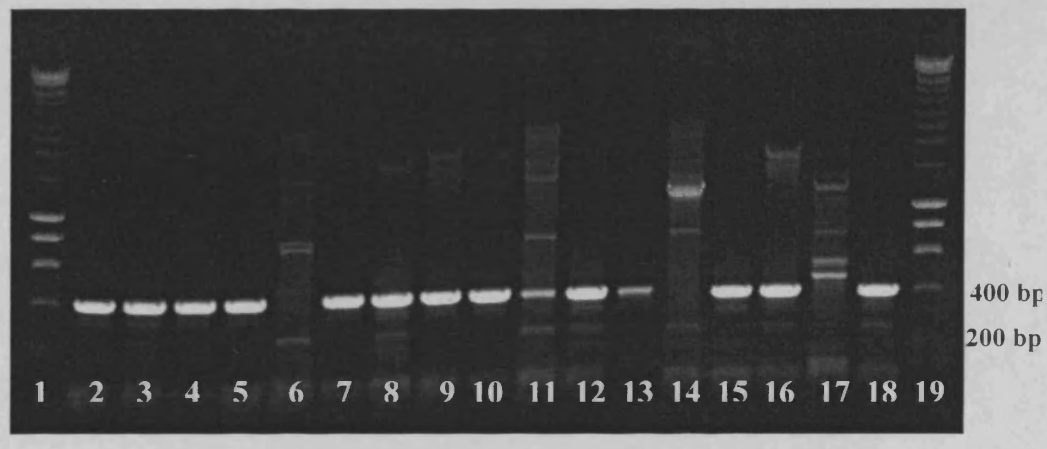

Figure B.4 Multiplex PCR experiment to detect the incidence of CTX-M type ESBLs groups 1,2,8,9 and 26 in *K. pneumoniae* isolates. Lane1: Marker. Lane2: AES982. Lane3: AES983. Lane4: AES984. Lane5: AES985. Lane6: AES987. Lane7: AES994. Lane8: AES1001. Lane9: AES1004. Lane10: AES1004(1). Lane11: AES1013. Lane12: AES1025. Lane13: AES1026. Lane14: AES1028. Lane15: AES1029. Lane16: AES1036. Lane17: AES1052. Lane18: AES1053. Lane19. Lane19: Marker.

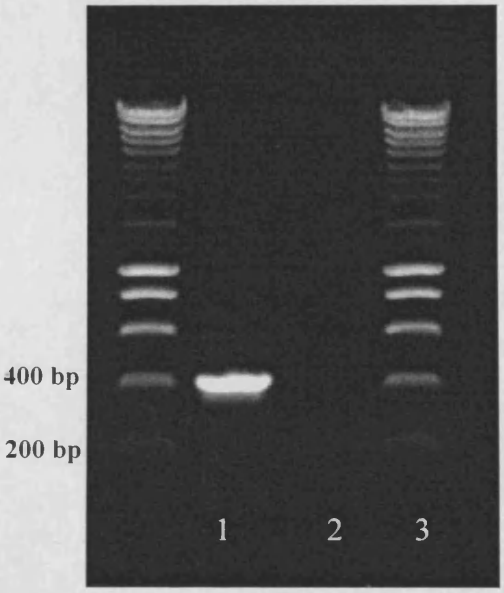

Figure B.5 Multiplex PCR experiment to detect the incidence of CTX-M type ESBLs groups 1,2,8,9 and 26 in *K. pneumoniae* isolates. Lane1: Marker. Lane2: Positive control AES140). Lane3: Negative control (H2O). Lane4: Marker

PFGE of *S*1 digestion of some isolates of *K. pneumoniae* and probing with $bla_{CTX\text{-}M\text{-}15}$ (Figures B.6 and B.7, next two pages)

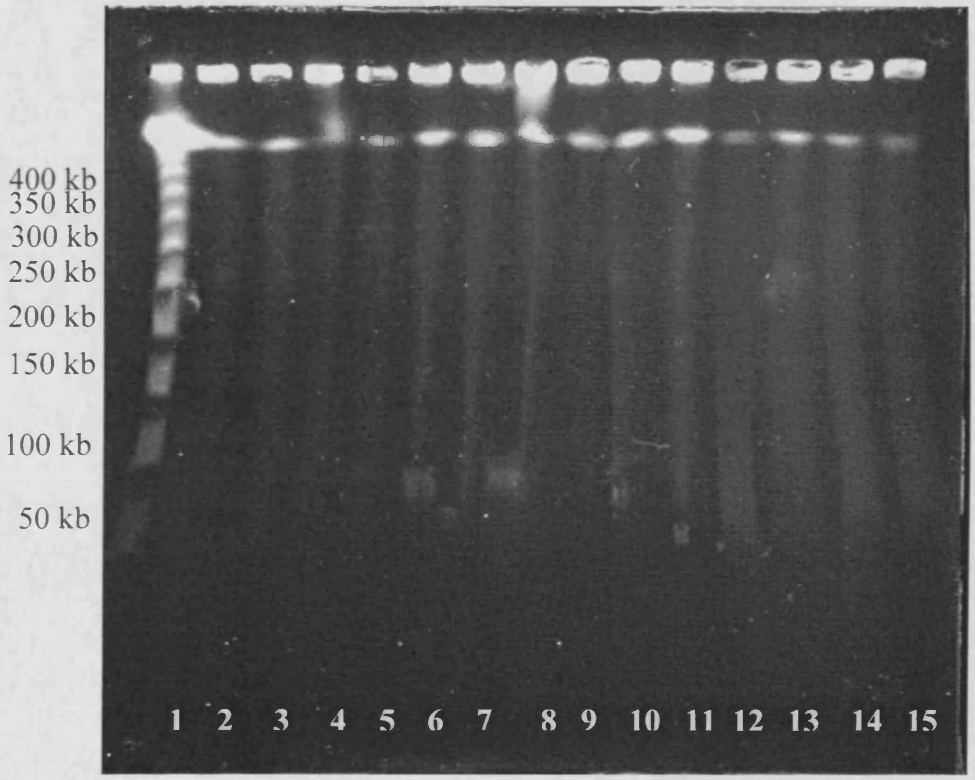

Figure B.6 PFGE of *S*1 digests for *K. pneumoniae* AES isolates. Lane1: Marker. Lane2: AES809. Lane3: AES817. Lane4: AES203. Lane5: AES836. Lane6: AES939. Lane7: AES961. Lane8: AES942. Lane9: AES188. Lane10: AES994. Lane11: AES960. Lane12: AES970. Lane13: AES975. Lane14: AES977. Lane15: AES982.

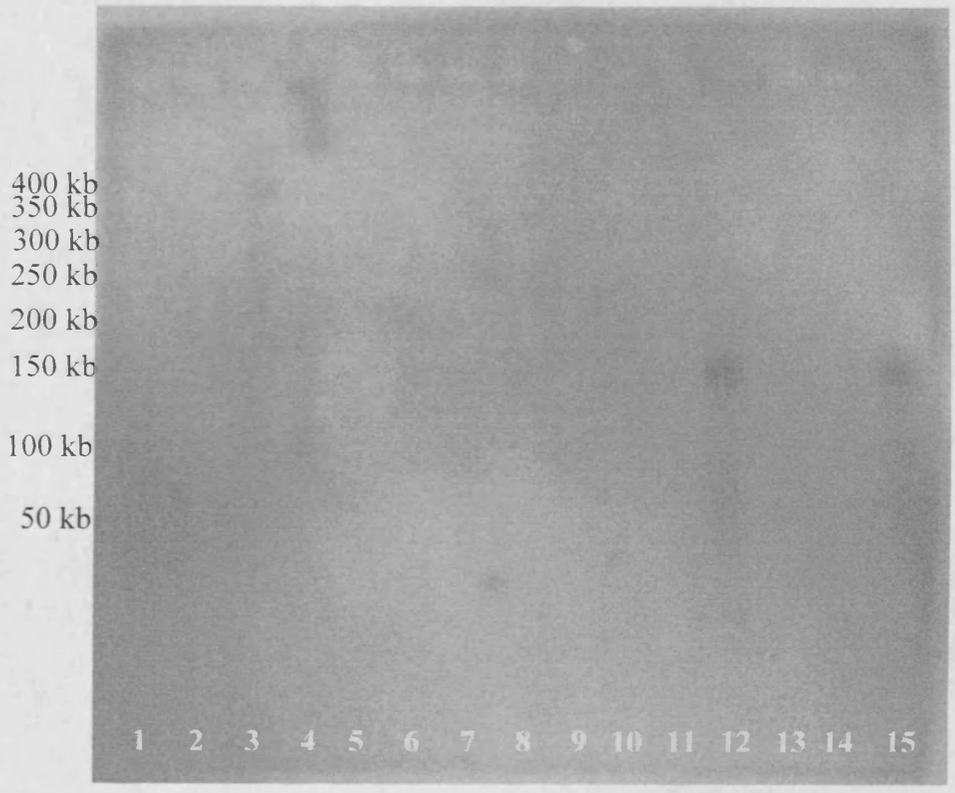

Figure B.7 Autorad after probing with *bla*$_{\text{CTX-M-15}}$/IS*Ecp1* of blotted PFGE from fig B6. Lane1: Marker. Lane2: AES809. Lane3: AES817. Lane4: AES203. Lane5: AES836. Lane6: AES939. Lane7: AES961. Lane8: AES942. Lane9: AES188. Lane10: AES994. Lane11: AES960. Lane12: AES970. Lane13: AES975. Lane14: AES977. Lane15: AES982.

DNA sequences from class 1 integrons of some of *K. pneumoniae*
Figure legend is above the figure.

Figure B.8 DNA sequence of *K. pneumoniae* AES59 amplified by VAF primer.

AACCTTGACCGAACGCAGCGGTGGTAACGGCGCAG
TGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCAT
CCA
AGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGA
TGTTA
CGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAGGGAGTTAAATTGA
AAATAT
CATTGATTTCTGCAGTGTCAGAAAATGGCGTAATCGGTAGTGGTCCTGATATCCC
GTGGT
CAGTAAAAGGTGAGCAACTACTCTTTAAAGCGCTCACATATAATCAATGGCTCCT
TGTCG
GAAGAAAAACATTTGACTCTATGGGTGTTCTTCCAAATCGCAAATATGCAGTAGT
GTCAA
AGAACGGAATTTCAAGCTCAAATGAAA

Figure B.9 Alignment of DNA from figure B.8 with DNA from gene bank Matched withDfrA17

```
>>EM_PRO:FJ895301 FJ895301.1 Shigella flexneri plasmid
unknown
clone 05100 class 1 integron DNA integrase intI1 (intI1),
dihydrofolate reductase DfrA17 (dfrA17), and
aminoglycoside-3'-adenylyltransferase (aadA5) genes,
complete cds. (2813 nt)
initn: 2110 init1: 2110 opt: 2110 Z-score: 2178.0 bits:
414.8 E(142439246): 7.8e-112
banded Smith-Waterman score: 2110; 100.0% identity (100.0%
similar) in 422 nt overlap (1-422:1063-1484)

10 20 30
EMBOSS AACCTTGACCGAACGCAGCGGTGGTAACGG
::::::::::::::::::::::::::::::
EM_PRO
GTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAAC
GG
1040 1050 1060 1070 1080 1090

40 50 60 70 80 90
EMBOSS
CGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGG
GC
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::
EM_PRO
```

```
CGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTTGTACAGTCTATGCCTCGG
GC
1100 1110 1120 1130 1140 1150

100 110 120 130 140 150
EMBOSS
ATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAAC
GA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::
EM_PRO
ATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAAC
GA
1160 1170 1180 1190 1200 1210

160 170 180 190 200 210
EMBOSS
TGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAGGGAGTTAAATTG
AA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::
EM_PRO
TGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAGGGAGTTAAATTG
AA
1220 1230 1240 1250 1260 1270

220 230 240 250 260 270
EMBOSS
AATATCATTGATTTCTGCAGTGTCAGAAAATGGCGTAATCGGTAGTGGTCCTGATATC
CC
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::
EM_PRO
AATATCATTGATTTCTGCAGTGTCAGAAAATGGCGTAATCGGTAGTGGTCCTGATATC
CC
1280 1290 1300 1310 1320 1330

280 290 300 310 320 330
EMBOSS
GTGGTCAGTAAAAGGTGAGCAACTACTCTTTAAAGCGCTCACATATAATCAATGGCTC
CT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::
EM_PRO
GTGGTCAGTAAAAGGTGAGCAACTACTCTTTAAAGCGCTCACATATAATCAATGGCTC
CT
1340 1350 1360 1370 1380 1390

340 350 360 370 380 390
EMBOSS
TGTCGGAAGAAAAACATTTGACTCTATGGGTGTTCTTCCAAATCGCAAATATGCAGTA
GT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::
EM_PRO
TGTCGGAAGAAAAACATTTGACTCTATGGGTGTTCTTCCAAATCGCAAATATGCAGTA
GT
1400 1410 1420 1430 1440 1450

400 410 420
EMBOSS GTCAAAGAACGGAATTTCAAGCTCAAATGAAA
```

```
:::::::::::::::::::::::::::::::
EM_PRO
GTCAAAGAACGGAATTTCAAGCTCAAATGAAAACGTCCTAGTTTTTCCTTCAATAGAA
AA
1460 1470 1480 1490 1500 1510

EM_PRO
TGCTTTGAAAGAGCTATCAAAAGTTACAGATCATGTATATGTCTCTGGCGGGGGTCAA
AT
1520 1530 1540 1550 1560 1570
```

2	EM_PRO:FN568351	Kluyvera georgiana conjugative IncFII plasmid pTC10 (partial) harboring a class 1 integron (dfrA17 and aadA5 gene cassettes), Tn3-bla(TEM-1b)-IS26 and Tn21 (partial) *Cross-references and related information in:* • Nucleotide Sequences • Protein Families • Ontologies • Protein Sequences	10872	2110	100.0	100.0	1

```
>>EM_PRO:FN568351 FN568351.1 Kluyvera georgiana
conjugative
IncFII plasmid pTC10 (partial) harboring a class 1
integron
(dfrA17 and aadA5 gene cassettes), Tn3-bla(TEM-1b)-
IS26 and
Tn21 (partial) (10872 nt)
initn: 2110 init1: 2110 opt: 2110 Z-score: 2165.2
bits: 414.3 E(142439246): 1e-111
banded Smith-Waterman score: 2110; 100.0% identity
(100.0% similar) in 422 nt overlap (1-422:6602-7023)

10 20 30
EMBOSS AACCTTGACCGAACGCAGCGGTGGTAACGG
:::::::::::::::::::::::::::::::
EM_PRO
GTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGG
TAACGG
6580 6590 6600 6610 6620 6630

40 50 60 70 80 90
EMBOSS
CGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCC
TCGGGC
::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::::::
EM_PRO
CGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCC
TCGGGC
6640 6650 6660 6670 6680 6690

100 110 120 130 140 150
EMBOSS
ATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAG
CAACGA
```

```
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::::::
EM_PRO
ATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAG
CAACGA
6700 6710 6720 6730 6740 6750

160 170 180 190 200 210
EMBOSS
TGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAGGGAGTTAA
ATTGAA
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::::::
EM_PRO
TGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAGGGAGTTAA
ATTGAA
6760 6770 6780 6790 6800 6810

220 230 240 250 260 270
EMBOSS
AATATCATTGATTTCTGCAGTGTCAGAAAATGGCGTAATCGGTAGTGGTCCTGA
TATCCC
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::::::
EM_PRO
AATATCATTGATTTCTGCAGTGTCAGAAAATGGCGTAATCGGTAGTGGTCCTGA
TATCCC
6820 6830 6840 6850 6860 6870

280 290 300 310 320 330
EMBOSS
GTGGTCAGTAAAAGGTGAGCAACTACTCTTTAAAGCGCTCACATATAATCAATG
GCTCCT
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::::::
EM_PRO
GTGGTCAGTAAAAGGTGAGCAACTACTCTTTAAAGCGCTCACATATAATCAATG
GCTCCT
6880 6890 6900 6910 6920 6930

340 350 360 370 380 390
EMBOSS
TGTCGGAAGAAAAACATTTGACTCTATGGGTGTTCTTCCAAATCGCAAATATGC
AGTAGT
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
::::::
EM_PRO
TGTCGGAAGAAAAACATTTGACTCTATGGGTGTTCTTCCAAATCGCAAATATGC
AGTAGT
6940 6950 6960 6970 6980 6990

400 410 420
EMBOSS GTCAAAGAACGGAATTTCAAGCTCAAATGAAA
::::::::::::::::::::::::::::::::
EM_PRO
GTCAAAGAACGGAATTTCAAGCTCAAATGAAAACGTCCTAGTTTTTCCTTCAAT
AGAAAA
7000 7010 7020 7030 7040 7050

EM_PRO
TGCTTTGAAAGAGCTATCAAAAGTTACAGATCATGTATATGTCTCTGGCGGGGG
```

```
TCAAAT
7060 7070 7080 7090 7100 7110
```

Figure B. 10 DNA sequence of *K. pneumoniae* AES59 1.5 kb amplified by QacR primer

AGCCNGCCTTTCTGATATATCTCCCAATTTGTGTAGGGCTTATTATG
CACGCTTAAAAATAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATT
ATGTG
CTTAGTGCATCTAACGCATAGTTGAGCGGCGGGCGCAGCCCGTCCGCTTGAACGC
CGAGT
TAGGCATCAGATGCCCTCGGCGCGGGTCGATGCACTTTTCGCACATGCCGCTCAA
CGCAA
GATTCTCTCAATCGTTGCTTTGGCATATCGAACGAACGCGGCCGTCTCTTCGACGC
GCAT
TGCTAGGTCGTCGTCCTCGCTACCCAGGTACGCCGCGCGTGCCTTGCAGATGAGG
GGCCG
ATGCTCGGCAGGCAAACGCTCCGATACCCATGCGGCAGCAACGTCCTTAGGAGCA
ATGAG
ACCAGTTGAAGCGCTGTACCAAATGCGAGCAAGAGCAAGAACGACGTTCCGCTC
GTCACC
CTTCCAATCCGACTCTGCATTCCACTGGGCAATAGTGTCGAAAAGCGCCTTGGAN
AAATG
CTCCTTCGGCNCCGGCTCGAAAAAC

Figure B.11 Alignment of DNA sequence from figure B.10 with DNA from gene bank.

Matched with aadA5

```
>  gb|EU914101.1|  Escherichia coli strain 59 class 1
integron, complete sequence;
ethidium bromide and quaternary ammonium compound export
protein (qacEdelta1), dihydropteroate synthase type 1 Sul1
(sul1),
hypothetical protein, and chromate transport protein
ChrA (chrA) genes, complete cds; and insertion sequence IS26,
partial sequence
Length=4828

 Score = 1003 bits (543),  Expect = 0.0
 Identities = 549/553 (99%), Gaps = 1/553 (0%)
 Strand=Plus/Minus

Query  1     AGCCNGCCTTTC-
TGATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAA  59
              |||| |||||||
||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1730
AGCCAGCCTTTCATGATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAA
1671
```

```
Query  60
TAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCT
119

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1670
TAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCT
1611

Query  120
AACGCATAGTTGAGCGGCGGGCGCAGCCCGTCCGCTTGAACGCCGAGTTAGGCATCAGAT
179

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1610
AACGCATAGTTGAGCGGCGGGCGCAGCCCGTCCGCTTGAACGCCGAGTTAGGCATCAGAT
1551

Query  180
GCCCTCGGCGCGGGTCGATGCACTTTTCGCACATGCCGCTCAACGCAAGATTCTCTCAAT
239

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1550
GCCCTCGGCGCGGGTCGATGCACTTTTCGCACATGCCGCTCAACGCAAGATTCTCTCAAT
1491

Query  240
CGTTGCTTTGGCATATCGAACGAACGCGGCCGTCTCTTCGACGCGCATTGCTAGGTCGTC
299

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1490
CGTTGCTTTGGCATATCGAACGAACGCGGCCGTCTCTTCGACGCGCATTGCTAGGTCGTC
1431

Query  300
GTCCTCGCTACCCAGGTACGCCGCGCGTGCCTTGCAGATGAGGGGCCGATGCTCGGCAGG
359

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1430
GTCCTCGCTACCCAGGTACGCCGCGCGTGCCTTGCAGATGAGGGGCCGATGCTCGGCAGG
1371

Query  360
CAAACGCTCCGATACCCATGCGGCAGCAACGTCCTTAGGAGCAATGAGACCAGTTGAAGC
419

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1370
CAAACGCTCCGATACCCATGCGGCAGCAACGTCCTTAGGAGCAATGAGACCAGTTGAAGC
1311

Query  420
GCTGTACCAAATGCGAGCAAGAGCAAGAACGACGTTCCGCTCGTCACCCTTCCAATCCGA
479

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  1310
GCTGTACCAAATGCGAGCAAGAGCAAGAACGACGTTCCGCTCGTCACCCTTCCAATCCGA
1251

Query  480
CTCTGCATTCCACTGGGCAATAGTGTCGAAAAGCGCCTTGGANAAATGCTCCTTCGGCNC
539
               ||||||||||||||||||||||||||||||||||||||||
|||||||||||||| |
Sbjct  1250
CTCTGCATTCCACTGGGCAATAGTGTCGAAAAGCGCCTTGGAGAAATGCTCCTTCGGCAC
1191

Query  540   CGGCTCGAAAAAC  552
              |||||||||||||
Sbjct  1190  CGGCTCGAAAAAC  1178
```

Figure B.12 DNA sequence from *K. pneumoniae* AES135 (1kb) amplified VAF and QacR primer

```
CNGCCTTTCNGATATATCTCCCAATTTGTGTAGGGCTTATTAT
GCACGCTTAAAAATAATAAAAACAGACTTGACCTGATAGTTTGGCTGTGAGCAAT
TATGT
GCTTAGTGCATCTAACGCCGCTATCAATTGCGGTAAAAAGCGTAGTGAGCGCGGC
GAACG
AAGCTTTTTGCCGTCAATTGCATAGCTTTGTTAACCCTTTTTCCAAATTTGATAGC
AATA
GTTAATGTTTGAACTAAAATGTTGCTCAAAAACAACTTCNAAGAAGTTGGGAATA
TTCGG
GAAGAAAACATCCCCTTCTGGCTCAATGTCNATCGTCGATACNTGGAGCGTAGAG
GCCAT
GGGCAACGTTTCTCTGTAAATCTCCCCGCCACCAGACACTATAACGTGACCGGNG
ANNNN
NNNTAGACCGCCCATGGCCTCTTCGATCGACGGGAATNCTACTACGTTGTCNTTA
TTGGC
CGNCCANGCTGANCGAGTAACNNCCGNNNATTTCCTATTGGGGAGNGCCCCCNNT
GATNN
NNANNNNTTGCGGNCNNNCAN
```

Figure B.13 Alignment of DNA sequence from fig. B12with DNA from gene bank
Matched with dfrA30

```
>  gb|JN121384.1|  Acinetobacter baumannii strain RUH875
antibiotic resistance island
AbaR21, partial sequence
Length=1789

 Score =  534 bits (289),  Expect = 2e-148
 Identities = 351/386 (91%), Gaps = 5/386 (1%)
 Strand=Plus/Minus
```

```
Query  13    AGCCNGCCTTTC-
TGATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAA  71
              ||||  |||||||
|||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1044
AGCCAGCCTTTCATGATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAA
985

Query  72
TAATAAAAACAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCT
131
              ||||||||
|||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  984
TAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCT
925

Query  132
AACGCCGCTATCAATTGCGGTAAAAAGCGTAGTGAGCGCGGCGAACGAAGCTTTTTGCCG
191
              ||||||| ||||||||||||||| ||||||||  ||||
|||||||||||||||| |||
Sbjct  924
AACGCCGTTATCAATTGCGGTAAGAAGCGTAGCAAGCGAAGCGAACGAAGCTTTTTACCG
865

Query  192
TCAATTGCATAGCTTTGTTAACCCTTTTTCCAAATTTGATAGCAATAGTTAATGTTTGAA
251
              ||||||||||||||||||||||||||||
|||||||||||||||||||||||||||||
Sbjct  864
TCAATTGCATAGCTTTGTTAACCCTTTTGCCAAATTTGATAGCAATAGTTAATGTTTGAG
805

Query  252   CTAAAATGTTGCTCAAAAACAACTTCGAAGA-
ANTTGGGAATATTCGGGAAGAAAACATC  310
              |||||  |||||||||||||||||||||||  | ||||||||||||
||||||||||
Sbjct  804   CTAAAGTGTTGCTCAAAAACAACTTCGAAGGTA-
TTGGGAATATTCGGAAAGAAAACATC  746

Query  311   CCCTTCTGGCTCAATGTCGATCGNNNATACATGNANCGTANAGGNCC-
TGGNNAACGTTT  369
               |||||  ||||||||  ||  ||||   |||  |||  |  ||||  |||  ||
|||  ||  ||||
Sbjct  745   TCCTTCCGGCTCAATATCAATCGTCGATATATGGAGCGTAGAGG-
CCATGGGCAATGTTT  687

Query  370   CTCTGNAAATCTCCCCGCCNCCAGAC  395
              |||||  ||||||||||||  ||||||
Sbjct  686   CTCTGTAAATCTCCCCGCCACCAGAC  661
```

Figure B.14 DNA sequence from *K. pneumoniae* AES48 amplified by QacR primer
(1.5 kb)

GCCNGCCTTTCNGATATATCTCCCNATTTGTGTAGGGCTTATTAT
GCACGCTTAAAAATAATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAAT
TATGT
GCTTAGTGCATCTAACGCCGGAGTTAAGCCGCCGCGCGTAGCGCGGTCGGCTTGA
ACGAA
TTGTTAGACATCATTTACCAACTGACTTGATGATCTCGCCTTTCACAAAGCGAATA
AATT
CTTCCAAGTGATCTGCGCGTGAGGCCAAGTGATCTTCTTTTTGTCCCAGATAAGCT
TGCT
TAGCTTCAAGTAAGACGGGCTGATACTGGGCAGGTAGGCGTTTTATTGCCCAGTC
GGCAG
CGACATCCTTCGGCGCGATTTTGCCGGNTATTGCGCTGTACCAAATGCGGGACAA
CGTAA
GCACTACATTTCGCTCATCGCCGGCCCAGTCGGGCTGCGAGTTCCATAGCTTCAA
GGTTT
CCCTCANCGCCTCNAATANATCCTGTTCAGGAANCGGGTCAAAGAATTCCTCCGN
TGCCG
GACCTACCNAGG

Figure B.15 Alignment of DNA sequence from fig. B.14 with DNA from gene bank.
Matched with aadA2

```
>  dbj|AP012208.1|  Escherichia coli plasmid pNDM-1_Dok01
DNA, complete sequence,
strain: NDM-1 Dok01
Length=195560

 Score =  950 bits (514),  Expect = 0.0
 Identities = 527/538 (98%), Gaps = 1/538 (0%)
 Strand=Plus/Minus

Query  1       GCCNGCCTTTC-
NGATATATCTCCCNATTTGTGTAGGGCTTATTATGCACGCTTAAAAAT  59
               ||| |||||||  ||||||||||||
||||||||||||||||||||||||||||||||
Sbjct  115050
GCCAGCCTTTCATGATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAAT
114991

Query  60
AATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTA
119

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114990
AATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTA
114931

Query  120
ACGCCGGAGTTAAGCCGCCGCGCGTAGCGCGGTCGGCTTGAACGAATTGTTAGACATCAT
179

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  114930
ACGCCGGAGTTAAGCCGCCGCGCGTAGCGCGGTCGGCTTGAACGAATTGTTAGACATCAT
114871

Query  180
TTACCAACTGACTTGATGATCTCGCCTTTCACAAAGCGAATAAATTCTTCCAAGTGATCT
239

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114870
TTACCAACTGACTTGATGATCTCGCCTTTCACAAAGCGAATAAATTCTTCCAAGTGATCT
114811

Query  240
GCGCGTGAGGCCAAGTGATCTTCTTTTTGTCCCAGATAAGCTTGCTTAGCTTCAAGTAAG
299

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114810
GCGCGTGAGGCCAAGTGATCTTCTTTTTGTCCCAGATAAGCTTGCTTAGCTTCAAGTAAG
114751

Query  300
ACGGGCTGATACTGGGCAGGTAGGCGTTTTATTGCCCAGTCGGCAGCGACATCCTTCGGC
359

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114750
ACGGGCTGATACTGGGCAGGTAGGCGTTTTATTGCCCAGTCGGCAGCGACATCCTTCGGC
114691

Query  360
GCGATTTTGCCGGNTATTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGC
419
                ||||||||||||
||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114690
GCGATTTTGCCGGTTATTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGC
114631

Query  420
TCATCGCCGGCCCAGTCGGGCTGCGAGTTCCATAGCTTCAAGGTTTCCCTCANCGCCTCN
479

|||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  114630
TCATCGCCGGCCCAGTCGGGCTGCGAGTTCCATAGCTTCAAGGTTTCCCTCAGCGCCTCG
114571

Query  480
AATANATCCTGTTCAGGAANCGGGTCAAAGAATTCCTCCGNTGCCGGACCTACCNAGG  537
                ||||  |||||||||||||| ||||||||||||||||||||
||||||||||||| |||
Sbjct  114570
AATAGATCCTGTTCAGGAACCGGGTCAAAGAATTCCTCCGCTGCCGGACCTACCAAGG
114513
```

>gb|HQ730120.1| Escherichia coli strain WM31a01 insertion sequence IS26, resolvase

```
(tnpR) gene, and transposon Tn1721, complete sequence;
TnpM gene, complete cds; and class 1 integron, partial sequence
Length=4988

 Score =  950 bits (514),  Expect = 0.0
 Identities = 527/538 (98%), Gaps = 1/538 (0%)
 Strand=Plus/Minus

Query  1     GCCNGCCTTTC-
NGATATATCTCCCNATTTGTGTAGGGCTTATTATGCACGCTTAAAAAT  59
              ||| |||||||  ||||||||||||
|||||||||||||||||||||||||||||||||
Sbjct  4710
GCCAGCCTTTCATGATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAAT
4651

Query  60
AATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTA
119

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4650
AATAAAAGCAGACTTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTA
4591

Query  120
ACGCCGGAGTTAAGCCGCCGCGCGTAGCGCGGTCGGCTTGAACGAATTGTTAGACATCAT
179

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4590
ACGCCGGAGTTAAGCCGCCGCGCGTAGCGCGGTCGGCTTGAACGAATTGTTAGACATCAT
4531

Query  180
TTACCAACTGACTTGATGATCTCGCCTTTCACAAAGCGAATAAATTCTTCCAAGTGATCT
239

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4530
TTACCAACTGACTTGATGATCTCGCCTTTCACAAAGCGAATAAATTCTTCCAAGTGATCT
4471

Query  240
GCGCGTGAGGCCAAGTGATCTTCTTTTTGTCCCAGATAAGCTTGCTTAGCTTCAAGTAAG
299

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4470
GCGCGTGAGGCCAAGTGATCTTCTTTTTGTCCCAGATAAGCTTGCTTAGCTTCAAGTAAG
4411

Query  300
ACGGGCTGATACTGGGCAGGTAGGCGTTTTATTGCCCAGTCGGCAGCGACATCCTTCGGC
359

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4410
ACGGGCTGATACTGGGCAGGTAGGCGTTTTATTGCCCAGTCGGCAGCGACATCCTTCGGC
4351
```

```
Query  360
GCGATTTTGCCGGNTATTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGC
419
               ||||||||||||
||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4350
GCGATTTTGCCGGTTATTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGC
4291

Query  420
TCATCGCCGGCCCAGTCGGGCTGCGAGTTCCATAGCTTCAAGGTTTCCCTCANCGCCTCN
479

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  4290
TCATCGCCGGCCCAGTCGGGCTGCGAGTTCCATAGCTTCAAGGTTTCCCTCAGCGCCTCG
4231

Query  480
AATANATCCTGTTCAGGAANCGGGTCAAAGAATTCCTCCGNTGCCGGACCTACCNAGG  537
               ||||| |||||||||||||| ||||||||||||||||||||
||||||||||||| |||
Sbjct  4230
AATAGATCCTGTTCAGGAACCGGGTCAAAGAATTCCTCCGCTGCCGGACCTACCAAGG
4173
```

Figure B.16 DNA sequence from *K. pneumoniae* AES48 amplified by VAF primer
(1.5 kb)

```
NNNNNNNNNCNNNNNNCACTGNNNNNNNNCTTGACCGAACGCAGCGGTGGTAACG
GCGCAG
TGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCAT
CCA
AGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGA
TGTTA
CGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATATGAACTCGGAATCAGT
ACGCAT
TTATCTCGTTGCTGCGATGGGAGCCAATCGGGTTATTGGCAATGGTCCTAATATCC
CCTG
GAAAATTCCGGGTGAGCAGAAGATTTTTCGCAGACTCACTGAGGGAAAAGTCGTT
GTCAT
GGGGCGAAAGACCTTTGAGTCTATCGGCAAGCCTCTACCGAACCGTCACACATTG
GTAAT
CTCACGCCAAGCTAANTACCGCGCCACTGGNTGCGTAGTTGTTTCAACGCTGTCG
CACGC
TATCGCTTTGGCATCCGAACTCGGNAATGAANTCTNCGTCNNGGGNGGAGCNGAG
NNANA
NACTCTGGCACTACCT
```

Figure B.17 Alignment of DNA sequence from fig. B.16 with DNA sequences from gene bank. Matched with dfrA12 dihydrofolate reductase

```
>>EM_PRO:DQ390454; DQ390454 Escherichia coli strain 517-  (63946 nt)
rev-comp initn: 2562 init1: 2562 opt: 2562  Z-score: 2828.1  bits: 540.0
```

```
E(): 7.4e-151
banded Smith-Waterman score: 2562; 97.5% identity (97.5% similar) in 528
nt overlap (556-29:25146-25673)

                                          550       540       530
Seque-                                  AGGTAGTGCCAGAGTNTNTNNCTCNGCTCC
                                        :::::::::::::::: : :  ::: :::::
EM_PRO TACCTCAGATAGAAACACGCCGTGGGCGTGAGGTAGTGCCAGAGTGTATATCTCAGCTCC
       25120     25130     25140     25150     25160     25170

           520       510       500       490       480       470
Seque- NCCCNNGACGNAGANTTCATTNCCGAGTTCGGATGCCAAAGCGATAGCGTGCGACAGCGT
        :::  :::: ::: :::::: ::::::::::::::::::::::::::::::::::::::
EM_PRO GCCCGCGACGTAGAGTTCATTGCCGAGTTCGGATGCCAAAGCGATAGCGTGCGACAGCGT
       25180     25190     25200     25210     25220     25230

           460       450       440       430       420       410
Seque- TGAAACAACTACGCANCCAGTGGCGCGGTANTTAGCTTGGCGTGAGATTACCAATGTGTG
       :::::::::::::::: :::::::::::::: :::::::::::::::::::::::::::
EM_PRO TGAAACAACTACGCAGCCAGTGGCGCGGTAGTTAGCTTGGCGTGAGATTACCAATGTGTG
       25240     25250     25260     25270     25280     25290

           400       390       380       370       360       350
Seque- ACGGTTCGGTAGAGGCTTGCCGATAGACTCAAAGGTCTTTCGCCCCATGACAACGACTTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ACGGTTCGGTAGAGGCTTGCCGATAGACTCAAAGGTCTTTCGCCCCATGACAACGACTTT
       25300     25310     25320     25330     25340     25350

           340       330       320       310       300       290
Seque- TCCCTCAGTGAGTCTGCGAAAAATCTTCTGCTCACCCGGAATTTTCCAGGGGATATTAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TCCCTCAGTGAGTCTGCGAAAAATCTTCTGCTCACCCGGAATTTTCCAGGGGATATTAGG
       25360     25370     25380     25390     25400     25410

           280       270       260       250       240       230
Seque- ACCATTGCCAATAACCCGATTGGCTCCCATCGCAGCAACGAGATAAATGCGTACTGATTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ACCATTGCCAATAACCCGATTGGCTCCCATCGCAGCAACGAGATAAATGCGTACTGATTC
       25420     25430     25440     25450     25460     25470

           220       210       200       190       180       170
Seque- CGAGTTCATATGGCTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGAGTTCATATGGCTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTG
       25480     25490     25500     25510     25520     25530

           160       150       140       130       120       110
Seque- CTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGC
       25540     25550     25560     25570     25580     25590

           100        90        80        70        60        50
Seque- ATAGACTGTACAAAAAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ATAGACTGTACAAAAAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCA
       25600     25610     25620     25630     25640     25650

            40        30        20        10
Seque- CCGCTGCGTTCGGTCAAGNNNNNNNCAGTGNNNNNNGNNNNNNNNN
       :::::::::::::::::
```

```
EM_PRO CCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTAC
       25660     25670     25680     25690     25700     25710
```

```
>>EM_PRO:EU780013; EU780013 Klebsiella pneumoniae strain  (37606 nt)
 initn: 2562 init1: 2562 opt: 2562  Z-score: 2831.4  bits: 539.8 E():
8.1e-151
banded Smith-Waterman score: 2562; 97.5% identity (97.5% similar) in 528
nt overlap (29-556:8343-8870)

                10        20        30        40        50
Sequen   NNNNNNNNNCNNNNNNCACTGNNNNNNNCTTGACCGAACGCAGCGGTGGTAACGGCGC
                                       ::::::::::::::::::::::::::::
EM_PRO GCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGC
           8320      8330      8340      8350      8360      8370

       60        70        80        90       100       110
Sequen AGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATC
           8380      8390      8400      8410      8420      8430

      120       130       140       150       160       170
Sequen CAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGT
           8440      8450      8460      8470      8480      8490

      180       190       200       210       220       230
Sequen TACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATATGAACTCGGAATCAGTACGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATATGAACTCGGAATCAGTACGC
           8500      8510      8520      8530      8540      8550

      240       250       260       270       280       290
Sequen ATTTATCTCGTTGCTGCGATGGGAGCCAATCGGGTTATTGGCAATGGTCCTAATATCCCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ATTTATCTCGTTGCTGCGATGGGAGCCAATCGGGTTATTGGCAATGGTCCTAATATCCCC
           8560      8570      8580      8590      8600      8610

      300       310       320       330       340       350
Sequen TGGAAAATTCCGGGTGAGCAGAAGATTTTTCGCAGACTCACTGAGGGAAAAGTCGTTGTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TGGAAAATTCCGGGTGAGCAGAAGATTTTTCGCAGACTCACTGAGGGAAAAGTCGTTGTC
           8620      8630      8640      8650      8660      8670

      360       370       380       390       400       410
Sequen ATGGGGCGAAAGACCTTTGAGTCTATCGGCAAGCCTCTACCGAACCGTCACACATTGGTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ATGGGGCGAAAGACCTTTGAGTCTATCGGCAAGCCTCTACCGAACCGTCACACATTGGTA
           8680      8690      8700      8710      8720      8730

      420       430       440       450       460       470
Sequen ATCTCACGCCAAGCTAANTACCGCGCCACTGGNTGCGTAGTTGTTTCAACGCTGTCGCAC
       ::::::::::::::::: ::::::::::::: :::::::::::::::::::::::::::
EM_PRO ATCTCACGCCAAGCTAACTACCGCGCCACTGGCTGCGTAGTTGTTTCAACGCTGTCGCAC
           8740      8750      8760      8770      8780      8790

      480       490       500       510       520       530
Sequen GCTATCGCTTTGGCATCCGAACTCGGNAATGAANTCTNCGTCNNGGGNGGAGCNGAGNNA
       :::::::::::::::::::::::::: :::::: ::: :::: ::: ::::: ::: :
```

```
EM_PRO GCTATCGCTTTGGCATCCGAACTCGGCAATGAACTCTACGTCGCGGGCGGAGCTGAGATA
            8800       8810       8820       8830       8840       8850

      540        550
Sequen NANACTCTGGCACTACCT
        : ::::::::::::::::
EM_PRO TACACTCTGGCACTACCTCACGCCCACGGCGTGTTTCTATCTGAGGTACATCAAACCTTC
            8860       8870       8880       8890       8900       8910
```

Figure B.18 DNA sequence from *K. pneumoniae* AES74 amplified by CTX-M-15 F primer

NNNNNNNNNNNNNNGTGCCGCTGTATGCGCAACGGCGGACGTACAGCAAAAAC
TTGCCG
AATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACACAGCAG
ATAATT
CGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCACCAGTAAAGT
GATGG
CCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGC
NAGTTG
AGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAANNACGTCNN
TGGGA
CNATGTCNCTGGCTGANCTTANCGCGGCCGCGCTACAGTACANNNATAACGTGNN
GATGA
NNAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCGACN
GCTGG
GANANNAANNGNTCCNNCNCGACCGNACCNAGCCNACNTTAANNNNNGNNNTTC
CGGGCG
ATCCGNGTGNTACNANTTCNGCTCGAGTAATGGAGCNCACTCCGCGGATTNNGNN
NATGG
GTATCGCNTTTNNNTGACNTCCAACGGNCNCNNCTGGNGAATTGNNTNANNGGTG
NTNNN
NNTNNTNNAGCGNNCATNNNNNCNGNNNNGNNNNCNNC

Figure B.19 Alignment of DNA sequence from fig. B.18 with DNA sequence from gene bank

```
>>EM_PRO:EU935739; EU935739 Escherichia coli strain A pl  (117536 nt)
 initn: 3785 init1: 3785 opt: 3785  Z-score: 3796.6  bits: 720.5 E(): 6.3e-205
banded Smith-Waterman score: 3785; 100.0% identity (100.0% similar) in 757 nt
overlap (1-757:63040-63796)

                                              10         20         30
Sequen                                    GACCAGAATCAGCGGCGCACGATCTTTTGG
                                          ::::::::::::::::::::::::::::::
EM_PRO TGCCTTAGGTTGAGGCTGGGTGAAGTAAGTGACCAGAATCAGCGGCGCACGATCTTTTGG
   63010      63020      63030      63040      63050      63060

               40         50         60         70         80         90
Sequen CCAGATCACCGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCCCCCAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CCAGATCACCGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCCCCCAC
   63070      63080      63090      63100      63110      63120

              100        110        120        130        140        150
Sequen AACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCAT
```

```
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCAT
   63130      63140      63150      63160      63170      63180

                160        170        180        190        200        210
Sequen CCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGATTCCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGATTCCG
   63190      63200      63210      63220      63230      63240

                220        230        240        250        260        270
Sequen CAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAATGGCGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAATGGCGGT
   63250      63260      63270      63280      63290      63300

                280        290        300        310        320        330
Sequen GTTTAACGTCGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGGGCGAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GTTTAACGTCGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGGGCGAA
   63310      63320      63330      63340      63350      63360

                340        350        360        370        380        390
Sequen CGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACGTTATC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACGTTATC
   63370      63380      63390      63400      63410      63420

                400        410        420        430        440        450
Sequen GCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTT
   63430      63440      63450      63460      63470      63480

                460        470        480        490        500        510
Sequen TTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAA
   63490      63500      63510      63520      63530      63540

                520        530        540        550        560        570
Sequen CAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGT
   63550      63560      63570      63580      63590      63600

                580        590        600        610        620        630
Sequen GCTGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCTGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTT
   63610      63620      63630      63640      63650      63660

                640        650        660        670        680        690
Sequen AATCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AATCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTG
   63670      63680      63690      63700      63710      63720

                700        710        720        730        740        750
Sequen TACGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TACGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGT
   63730      63740      63750      63760      63770      63780


Sequen CGCCATC
       :::::::
```

```
EM_PRO CGCCATCAGCGTGAACTGGCGCAGTGATTTTTTAACCATGGGATTCCTTATTCTGGAAGA
   63790      63800      63810      63820      63830      63840
```

Figure B.20 DNA sequence from *K. pneumoniae* AES140 amplified by CTX-M-15 F primer

NNNNNNNNNNNNNNTGTGCCGCTGTATGCGCAACGGCGGACGTACAGCAAAAAC
TTGCCG
AATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACACAGCAG
ATAATT
CGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCACCAGTAAAGT
GATGG
CCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGC
GAGTTG
AGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAA
TGGGA
CGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGC
GATGA
ATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCGACA
GCTGG
GAGACGAAACGTTCCNTCTCGACCGTACCGAGCCGACGTTAANNACCGCCNNNN
NGGGCG
ATCCGCGTGATACCNNTTCNNCTCGGGCANTGGCNCAAACTCTGCGGANNNTGAC
GCTGG
NNNNNNCATTNNNCGN

Figure B.21 Alignment of *bla*$_{CTX-M-15}$ gene from fig. B.20 with DNA sequences from gene bank

```
>>EM_PRO:EU935739; EU935739 Escherichia coli strain A pl  (117536 nt)
 initn: 3790 init1: 3790 opt: 3790  Z-score: 3815.4  bits: 724.0 E(): 5.6e-206
banded Smith-Waterman score: 3790; 100.0% identity (100.0% similar) in 758 nt
overlap (1-758:63040-63797)

                                              10        20        30
Sequen                                    GACCAGAATCAGCGGCGCACGATCTTTTGG
                                          ::::::::::::::::::::::::::::::
EM_PRO TGCCTTAGGTTGAGGCTGGGTGAAGTAAGTGACCAGAATCAGCGGCGCACGATCTTTTGG
   63010      63020      63030      63040      63050      63060

               40         50         60         70         80         90
Sequen CCAGATCACCGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCCCCCAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CCAGATCACCGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCCCCCAC
   63070      63080      63090      63100      63110      63120

              100        110        120        130        140        150
Sequen AACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCAT
   63130      63140      63150      63160      63170      63180

              160        170        180        190        200        210
Sequen CCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGATTCCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGATTCCG
   63190      63200      63210      63220      63230      63240
```

```
               220        230        240        250        260        270
Sequen CAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAATGGCGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAATGGCGGT
   63250      63260      63270      63280      63290      63300

               280        290        300        310        320        330
Sequen GTTTAACGTCGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGGGCGAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GTTTAACGTCGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGGGCGAA
   63310      63320      63330      63340      63350      63360

               340        350        360        370        380        390
Sequen CGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACGTTATC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACGTTATC
   63370      63380      63390      63400      63410      63420

               400        410        420        430        440        450
Sequen GCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTT
   63430      63440      63450      63460      63470      63480

               460        470        480        490        500        510
Sequen TTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAA
   63490      63500      63510      63520      63530      63540

               520        530        540        550        560        570
Sequen CAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGT
   63550      63560      63570      63580      63590      63600

               580        590        600        610        620        630
Sequen GCTGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCTGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTT
   63610      63620      63630      63640      63650      63660

               640        650        660        670        680        690
Sequen AATCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AATCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTG
   63670      63680      63690      63700      63710      63720

               700        710        720        730        740        750
Sequen TACGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TACGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGT
   63730      63740      63750      63760      63770      63780


Sequen CGCCATCA
       ::::::::
EM_PRO CGCCATCAGCGTGAACTGGCGCAGTGATTTTTTAACCATGGGATTCCTTATTCTGGAAGA
   63790      63800      63810      63820      63830      63840
```

Figure B.22 DNA sequence of IS*Ecp1* from *K. pneumoniae* AES140

NNNNNNNNNNNNANNAGCAGTCTANNNNNNNNNNNNNTANNNNNNTTTGAAGC
TAATAAA
AAACACACGTGGAATTTAGGTTTCATTCTGGCGACGTCCGTATTNGCCTTTCGGAA
GCAT
AAAATCGGACGCGTTGTGGCTCGCTTCAGGTAAAATATTGACTATTCNNGTTGTT
GTTAT
TTCGTCTCTTCCAGAATAAGGAATCCCATGGTTAAAAAATCACTGCGCCAGTTCA
CGCTG
ATGGCGACGGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAA
CGGCG
GACGTACAGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGT
GTGGCA
TTGATTAACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGC
GATG
TGCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGC
GAACCG
AATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATC
CGATT
GCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCG
CTACAG
TACAGCGATAACNNNNNNAAAAAAAANNNANAAAANNNNNNNNNTGNNNNNN
NNCNGGG

Figure B.23 Alignment of DNA sequence from fig. B.22 with DNA from gene bank
Matched with IS*Ecp1*

```
>>EM_PRO:EU935740; EU935740 Escherichia coli strain C pl  (93732 nt)
rev-comp initn: 4018 init1: 3840 opt: 4197  Z-score: 3885.7  bits: 736.9 E():
7.1e-210
banded Smith-Waterman score: 4197; 96.8% identity (96.8% similar) in 893 nt
overlap (895-3:7081-7967)

                                 900       890       880       870
Seque-                             TATTTTTTNNTTGNTNNNAAAGTTTGANTTCCTTN
                                         : :  ::: :    :::::::: ::::::
EM_PRO TGTTTCAAATGATGATGCTTTCATATAACCTATTTTTGTTGTTCAAGTTTGA-TTCCTT-
             7060      7070      7080      7090      7100

          860       850       840       830       820       810
Seque- GGANTNNTTTCAGAATACAGACAGCAAATAAAGACCTTTCGTTTGAAGGTATGTATTTCT
       ::: :   :::::::::::::::::::::::::::::::::::::::: ::::::::::::
EM_PRO GGACTC--TTCAGAATACAGACAGCAAATAAAGACCTTTCGTTTGAA-GTATGTATTTCT
     7110        7120      7130      7140      7150       7160

          800       790       780       770       760       750
Seque- TGCAGCAAAAAATAATCAAAACCGCAAGATATGTAATCATGAAGTTGTCGGAAAACTATC
       :::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TGCAGC-AAAAATAATCAAAACCGCAAGATATGTAATCATGAAGTTGTCGGAAAACTATC
        7170       7180      7190      7200      7210      7220

          740       730       720       710       700       690
Seque- CGTACAAGGGAGTGTATGAAAAATGTCTGGTATAATAAGAATATCATCAATAAAATTGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGTACAAGGGAGTGTATGAAAAATGTCTGGTATAATAAGAATATCATCAATAAAATTGAG
         7230      7240      7250      7260      7270      7280

          680       670       660       650       640       630
```

```
Seque- TGTTGCTCTGTGGATAACTTGCAGAGTTTATTAAGTATCATTGCAGCAAAGATGAAATCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TGTTGCTCTGTGGATAACTTGCAGAGTTTATTAAGTATCATTGCAGCAAAGATGAAATCA
         7290       7300       7310       7320       7330       7340

          620        610        600        590        580        570
Seque- ATGATTTATCAAAAATGATTGAAAGGTGGTTGTAAATAATGTTACAATGTGTGAGAAGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ATGATTTATCAAAAATGATTGAAAGGTGGTTGTAAATAATGTTACAATGTGTGAGAAGCA
         7350       7360       7370       7380       7390       7400

          560        550        540        530        520        510
Seque- GTCTAAATTCTTCGTGAAATAGTGATTTTTGAAGCTAATAAAAAACACACGTGGAATTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GTCTAAATTCTTCGTGAAATAGTGATTTTTGAAGCTAATAAAAAACACACGTGGAATTTA
         7410       7420       7430       7440       7450       7460

          500        490        480        470        460        450
Seque- GGTTTCATTCTGGCGACGTCCGTATTTGCCTTTCGGAAGCATAAAATCGGACGCGTTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GGTTTCATTCTGGCGACGTCCGTATTTGCCTTTCGGAAGCATAAAATCGGACGCGTTGTG
         7470       7480       7490       7500       7510       7520

          440        430        420        410        400        390
Seque- GCTCGCTTCAGGTAAAATATTGACTATTCATGTTGTTGTTATTTCGTCTCTTCCAGAATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCTCGCTTCAGGTAAAATATTGACTATTCATGTTGTTGTTATTTCGTCTCTTCCAGAATA
         7530       7540       7550       7560       7570       7580

          380        370        360        350        340        330
Seque- AGGAATCCCATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AGGAATCCCATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTC
         7590       7600       7610       7620       7630       7640

          320        310        300        290        280        270
Seque- ACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAAAACTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAAAACTT
         7650       7660       7670       7680       7690       7700

          260        250        240        230        220        210
Seque- GCCGAATTAGAGCNNNNGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACACAGCAGAT
       :::::::::::::    ::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACACAGCAGAT
         7710       7720       7730       7740       7750       7760

          200        190        180        170        160        150
Seque- AATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCNNNNNTAAAGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::     :::::::
EM_PRO AATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCACCAGTAAAGTG
         7770       7780       7790       7800       7810       7820

          140        130        120        110        100         90
Seque- ATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGCGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO ATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGCGA
         7830       7840       7850       7860       7870       7880

           80         70         60         50         40         30
Seque- GTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAAN
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAAT
         7890       7900       7910       7920       7930       7940

           20         10
Seque- GGGACGANGTCACNGGCNGAGCTAG
```

```
        ::::::: ::::: ::: :::::
EM_PRO GGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCG
         7950       7960       7970       7980       7990       8000
```

Figure B.24 DNA sequence from *K. pneumoniae* AES261 amplified by CTX-M-15 primer

GGGAGTGCGCGGCGCGCTAGCTCAGCCAGTGACATCGTCCCATTGA
CGTGCTTTTCCGCA
ATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTG
ATTTAACAGATTC
GGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTT
ACTGGTGCTGCAC
ATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTG
CTGTGTTAATCAAT
GCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTT
TTTGCTGTACGTCC
GCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGG
TTGCCGTCGCCATC
AGCGTGAACTGGCGAGCTGATTTTA

Figure B.25 Alignment of DNA sequence from fig. B.24 with DNA from gene bank
Matched with $bla_{\text{CTX-M-15}}$

```
emb|FR828676.1| Escherichia coli plasmid pCTX913 tnpA gene,
blaCTX-M-15 gene
and delta tnpA gene (partial), isolate 913
Length=2656

 Score =  678 bits (367),  Expect = 0.0
 Identities = 377/381 (99%), Gaps = 4/381 (1%)
 Strand=Plus/Minus

Query  7     GCGCGG-CGCGCT-
AGCTCAGCCAGTGACATCGTCCCATTGACGTGCTTTTCCGCAATCG  64
              |||||| ||||||
||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1312
GCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTTTTCCGCAATCG
1253

Query  65
GATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACAGATTCGGTT
124

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1252
GATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACAGATTCGGTT
1193

Query  125
CGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGCTGCACATCG
184
```

```
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1192
CGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGCTGCACATCG
1133

Query  185
CAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAATCAATGCCA
244

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1132
CAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAATCAATGCCA
1073

Query  245
CACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCG
304

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1072
CACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCG
1013

Query  305
TTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCG
364

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1012
TTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCG
953

Query  365   TGAACTGGCG-AGCTGATTTT  384
              |||||||||| || |||||||
Sbjct  952   TGAACTGGCGCAG-TGATTTT  933
```

Figure B.26 DNA sequence from *K. pneumoniae* AES817 amplified by CTX-M-15 primers

TGTTCTGTAGCGCGGCGCGCTAGCTCAGCCAGTGACATCGTCCCAT
TGACGTGCTTTTCC
GCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTC
GCTGATTTAACAGA
TTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCAC
TTTACTGGTGCTG
CACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTAT
CTGCTGTGTTAATC
AATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAA
GTTTTTGCTGTACG
TCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGA
CGGTTGCCGTCGCC
ATCAGCGTGAACTGGCAAAAATGATTTTTA

Figure B.27 Alignment of DNA sequence from fig. B.26 with DNA from gene bank
Matched with *bla*$_{\text{CTX-M-15}}$

```
gb|JF918433.1| Escherichia coli insertion sequence ISEcp1,
partial sequence;
and insertion sequence IS26 cefotaximase (blaCTX-M-15) gene,
partial cds
Length=808

 Score =  680 bits (368),  Expect = 0.0
 Identities = 384/391 (98%), Gaps = 3/391 (1%)
 Strand=Plus/Minus

Query  1    TGTTCTGTAGCGCGG-CGCGCT-
AGCTCAGCCAGTGACATCGTCCCATTGACGTGCTTTT  58
             ||| |||||||||| ||||||
||||||||||||||||||||||||||||||||||||
Sbjct  500
TGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTTTT
441

Query  59
CCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACA
118

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  440
CCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACA
381

Query  119
GATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGC
178

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  380
GATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGC
321

Query  179
TGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAA
238

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  320
TGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAA
261

Query  239
TCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTA
298

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260
TCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTA
201
```

```
Query  299
CGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCG
358

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  200
CGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCG
141

Query  359  CCATCAGCGTGAACTGGCAAAAATGATTTTT  389
            ||||||||||||||||||  |  ||||||||
Sbjct  140  CCATCAGCGTGAACTGGCGCAG-TGATTTTT  111
```

Figure B.28 DNA sequence from *K. pneumoniae* AES984 amplified by CTX-M-15 primers

TGGAAGTAATACCTTGAAAGCGTGGTATGCTGAAACTATATCAAAG
AAGCCAAATACGAC
ATGGCGGTGGGTCATCTCTTGCTAAAGTCATTTTGGGCGAATGAAG
CCGTGTTTCAAATG
ATGATGCTTTCATATAACCTATTTTTGTTGTTCAAGTTTGATTCCTTG
GACTCTTCAGAA
TACAGACAGCAAATAAAGACCTTTCGTTTGAAGTATGTATTTCTTG
CAGCAAAAATAATC
AAAACCGCAAGATATGTAATCATGAAGTTGTCGGAAAACTATCCGT
ACAAGGGAGTGTAT
GAAAAATGTCTGGTATAATAAGAATATCATCAATAAAATTGAGTGT
TGCTCTGTGGATAA
CTTGCCGAGTACTTACCTATCATTGCTGCAACCATGAAATCCCTATT
GATTTAATAAAAA
ATGATTGAAAGGCGGTTGTAAATAATGTTACAATGTGGGAGAAGC
AGTCTAAATTCTTCG
TGAAATAGTGATTTTTGAAGCTAATAAAAAACACACGTGGAATTTA
GGGACTATTCATGT
TGTTGTTATTTCGTATCTTCCAGAATAAGGAATCCCATGGTTAAAA
AATCACTGCGCCAG
TTCACGCTGATGGCGACGGCAACCGTCACGCTGTTGTTAGGAAGTG
TGCCGCTGTATGCG
CAAACGGCGGACGTACAGCAAAAACTTGCCGATTTAGAGCGGCAG
TCGGGAGGCAGACTG
GGTGTGGCATGTGATTAACACGGCAGATGATTCGCAAATACTATAT
CGTGCTGATGAGCG
CTTTGCGATGTGCAGCACCAGTACAGTGATGGCCGCGGCCGCGATG
CTGAAAAGAAATGA
AAACAAACCGATCTGTTAAATCCGCGAGTTGACACCCAAAATCCG
ACCTTGTGACTATGA
CTCCCCATCGTGAAAGTCGCCTTGTGACATGTTTTGCGTGAGCTAC
GCTGTCGCGCTATT
ACACGTCCGCGGGGGTTTTTTTTTTATTTA

Figure B.29 Alignment of DNA sequence from fig. B.28 with DNA from gene bank
Matched with $bla_{\text{CTX-M-15}}$

```
Klebsiella pneumoniae strain C1865 TEM-1 beta-lactamase
(blaTEM-1)
gene, partial cds; TnpR (tnpR) gene, complete cds; insertion
sequence ISEcp1, complete sequence; CTX-M-15 extended-spectrum
beta-lactamase (blaCTX-M-15) and hypothetical protein
genes, complete cds; insertion sequence IS26, complete
sequence;
fluoroquinolone acetylating aminoglycoside-(6')-N-
acetyltransferase
(aac(6')-Ib-cr) gene, complete cds; and OXA-1
beta-lactamase (blaOXA-1) gene, partial cds
Length=8378

 Score = 1408 bits (762),  Expect = 0.0
 Identities = 850/890 (96%), Gaps = 15/890 (2%)
 Strand=Plus/Plus

Query  17    AAAGCGTGGT-ATGCTG-
AAACTATATCAAAGAAGCCAAATACGACATGGCGGTGGGTCA  74
              |||||||||| ||||||
||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2486
AAAGCGTGGTAATGCTGAAAACTATATCAAAGAAGCCAAATACGACATGGCGGTGGGTCA
2545

Query  75
TCTCTTGCTAAAGTCATTTTGGGCGAATGAAGCCGTGTTTCAAATGATGATGCTTTCATA
134

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2546
TCTCTTGCTAAAGTCATTTTGGGCGAATGAAGCCGTGTTTCAAATGATGATGCTTTCATA
2605

Query  135
TAACCTATTTTTGTTGTTCAAGTTTGATTCCTTGGACTCTTCAGAATACAGACAGCAAAT
194

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2606
TAACCTATTTTTGTTGTTCAAGTTTGATTCCTTGGACTCTTCAGAATACAGACAGCAAAT
2665

Query  195
AAAGACCTTTCGTTTGAAGTATGTATTTCTTGCAGCAAAAATAATCAAAACCGCAAGATA
254

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Sbjct  2666
AAAGACCTTTCGTTTGAAGTATGTATTTCTTGCAGCAAAAATAATCAAAACCGCAAGATA
2725

Query  255
TGTAATCATGAAGTTGTCGGAAAACTATCCGTACAAGGGAGTGTATGAAAAATGTCTGGT
314
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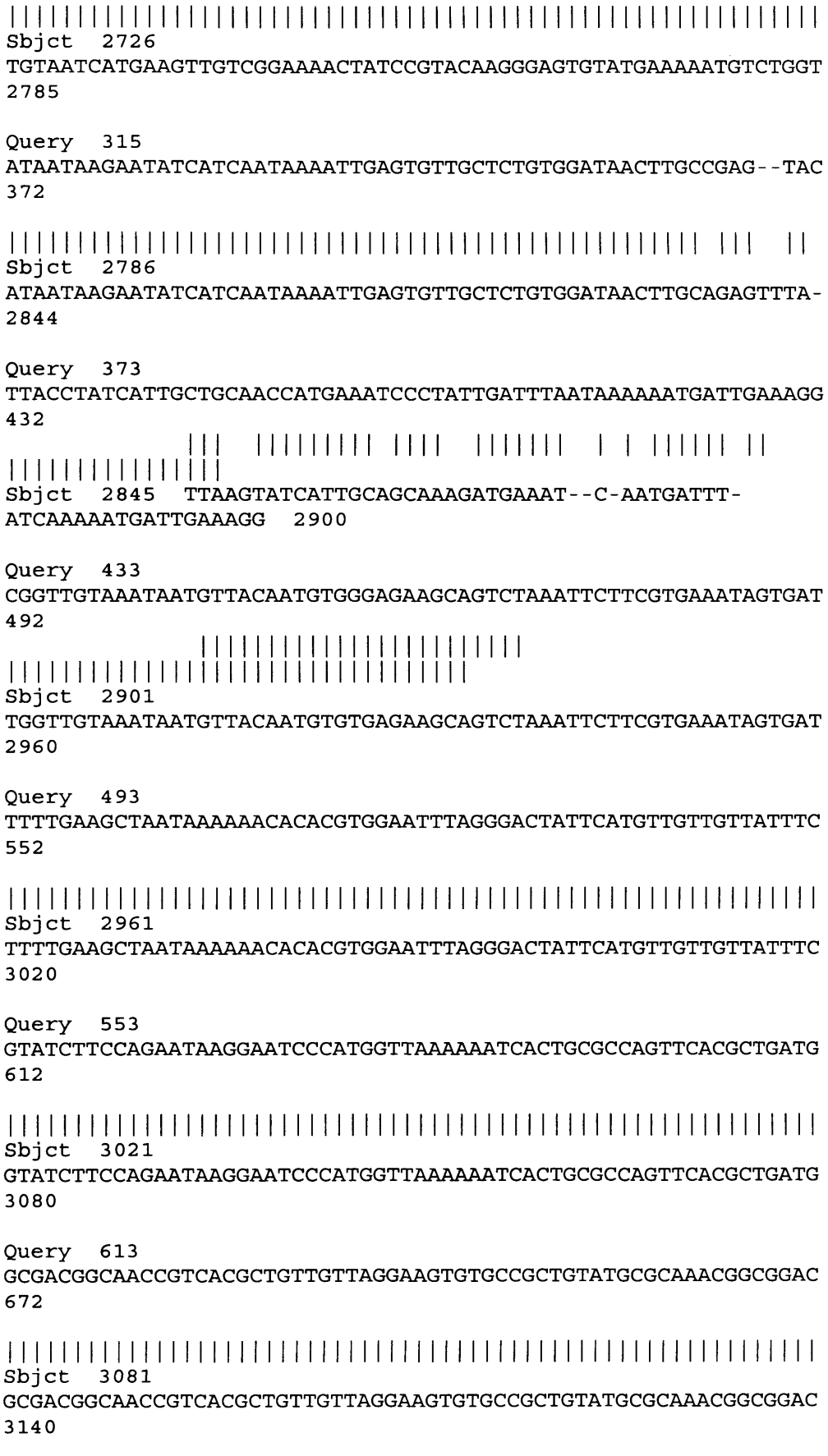
Sbjct 2726
TGTAATCATGAAGTTGTCGGAAAACTATCCGTACAAGGGAGTGTATGAAAAATGTCTGGT
2785
Query 315
ATAATAAGAATATCATCAATAAAATTGAGTGTTGCTCTGTGGATAACTTGCCGAG--TAC
372
Sbjct 2786
ATAATAAGAATATCATCAATAAAATTGAGTGTTGCTCTGTGGATAACTTGCAGAGTTTA-
2844
Query 373
TTACCTATCATTGCTGCAACCATGAAATCCCTATTGATTTAATAAAAAATGATTGAAAGG
432
Sbjct 2845 TTAAGTATCATTGCAGCAAAGATGAAAT--C-AATGATTT-
ATCAAAAATGATTGAAAGG 2900
Query 433
CGGTTGTAAATAATGTTACAATGTGGGAGAAGCAGTCTAAATTCTTCGTGAAATAGTGAT
492
Sbjct 2901
TGGTTGTAAATAATGTTACAATGTGTGAGAAGCAGTCTAAATTCTTCGTGAAATAGTGAT
2960
Query 493
TTTTGAAGCTAATAAAAAACACACGTGGAATTTAGGGACTATTCATGTTGTTGTTATTTC
552
Sbjct 2961
TTTTGAAGCTAATAAAAAACACACGTGGAATTTAGGGACTATTCATGTTGTTGTTATTTC
3020
Query 553
GTATCTTCCAGAATAAGGAATCCCATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATG
612
Sbjct 3021
GTATCTTCCAGAATAAGGAATCCCATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATG
3080
Query 613
GCGACGGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGAC
672
Sbjct 3081
GCGACGGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGAC
3140

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Query  673
GTACAGCAAAAACTTGCCGATTTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATGT
732
              ||||||||||||||||||||
|||||||||||||||||||||||||||||||||||||| |
Sbjct  3141
GTACAGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCAT-T
3199

Query  733
GATTAACACGGCAGATGATTCGCAAATACTATATCGTGCTGATGAGCGCTTTGCGATGTG
792
              ||||||||| |||||| ||||||||||||
||||||||||||||||||||||||||||
Sbjct  3200
GATTAACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTG
3259

Query  793   CAGCACCAGTACAGTGATGGCCGCGGCCGCGATGCTGAAAAGAAA-
TGAAAACAAACCGA  851
              |||||||||||| |||||||||||||||||| ||||||| | |||
|||||  | ||||||
Sbjct  3260  CAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGA-
AAAGTGAAAGCGAACCGA  3318

Query  852   -TCTGTTAAATCCGCGAGTTGACACCCAAAA-TCCGACCTTGTGA-CTAT
898
               |||||||||||| ||||||||| | | |||| || |||||||| | ||||
Sbjct  3319  ATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTAT
3368
```

Figure B.30 DNA sequence from *K. pneumoniae* AES1001 amplified by CTX-M-15 primers

TAAATGTTATGTGTGAGAGCAGTCTAAATTCTTCGTGAAATAGTGA
TTTTTGAAGCTAAT
AAAAAACACACGTGGAATTTAGGGACTATTCATGTTGTTGTTATTT
CGTATCTTCCAGAA
TAAGGAATCCCATGGTTAAAAAATCACTGCGCCAGTTCACGCTGAT
GGCGACGGCAACCG
TCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGA
CGTACAGCAAAAAC
TTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCAT
TGATTAACACAGCAG
ATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTG
CAGCACCAGTAAAG
TGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGA
ATCTGTTAAATCAGC
GAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGC
GGAAAAGCACGTCA
ATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTA
CAGCGATAACGTGG

CTGGTTAAATAAGCTTGTCTTTTGACCTTTCCATTGACGGGTTTTCC
ACCCGACTAAAAT
TTCAAGCGCAATATTTTTACTCCAACGATTTACGAGTAGTTCTTTCC
TTTTTTCAAACAA
CGCCGGGTCGGCCTTCATGGCGCTCCCACCCAATTGCCCACAAACT
ACCAAAAATTCGAA
TTTTTACCCGTTTAACAATGAAGCCAACTGCCCATCCCCCCATTTTC
TACTGATGTTTTT
TCTACCATCTCTTTCCTCACGCTGCTTTTTTTA

Figure B.31 Alignment of DNA sequence from fig. B.30 with DNA from gene bank
Matched with *bla*$_{\text{CTX-M-15}}$

```
Acinetobacter baumannii strain H1 hydroxyisourate hydrolase
gene,
complete cds; disrupted pyrimidine utilization transporter
gene, partial sequence; insertion sequence ISEcp1 transposase
(tnpA) gene, complete cds; CTX-M15 (blaCTX-M15) gene,
complete cds; disrupted orf477 gene, partial sequence;
transposon
Tn3 tnpA gene, partial sequence; and hypothetical protein
gene, complete cds
Length=5224

 Score =  981 bits (531),  Expect = 0.0
 Identities = 543/548 (99%), Gaps = 3/548 (1%)
 Strand=Plus/Plus

Query  9     ATGTGTGAG-
AGCAGTCTAAATTCTTCGTGAAATAGTGATTTTTGAAGCTAATAAAAAAC  67
              |||||||||
||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2615
ATGTGTGAGAAGCAGTCTAAATTCTTCGTGAAATAGTGATTTTTGAAGCTAATAAAAAAC
2674

Query  68
ACACGTGGAATTTAGGGACTATTCATGTTGTTGTTATTTCGTATCTTCCAGAATAAGGAA
127

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Sbjct  2675
ACACGTGGAATTTAGGGACTATTCATGTTGTTGTTATTTCGTATCTTCCAGAATAAGGAA
2734

Query  128
TCCCATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTCACGCT
187

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Sbjct  2735
TCCCATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTCACGCT
2794
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Query  188
GTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAAAACTTGCCGA
247

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2795
GTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAAAACTTGCCGA
2854

Query  248
ATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACACAGCAGATAATTC
307

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2855
ATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAACACAGCAGATAATTC
2914

Query  308
GCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCACCAGTAAAGTGATGGC
367

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2915
GCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCACCAGTAAAGTGATGGC
2974

Query  368
CGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGCGAGTTGA
427

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2975
CGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGCGAGTTGA
3034

Query  428
GATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGAC
487

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Sbjct  3035
GATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGAC
3094

Query  488
GATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCTGGTTA
547

|||||||||||||||||||||||||||||||||||||||||||||||||||| | | |
Sbjct  3095
GATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGC-GATGA
3153

Query  548   AATAAGCT  555
              | ||||||
Sbjct  3154  A-TAAGCT  3160
```

Figure B.32 Alignment of RpoB from *K. pneumoniae* AES817 sequence type as ST 511 with gene bank

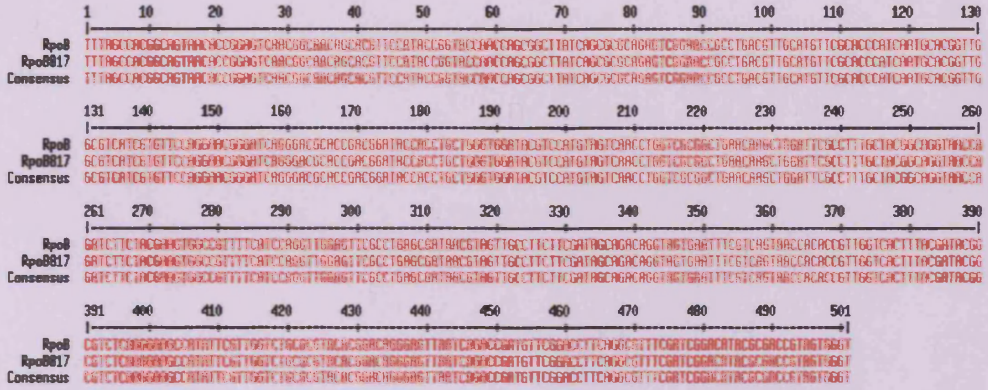

Figure B.33 Alignment of GapA from *K. pneumoniae* AES817 sequence type as ST 511 with gene bank

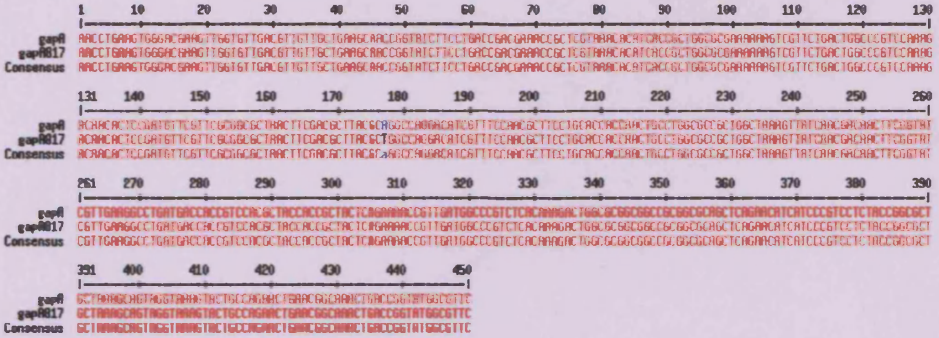

Figure B.34 Alignment of infB from *K. pneumoniae* AES817 sequence type as ST 511 with gene bank

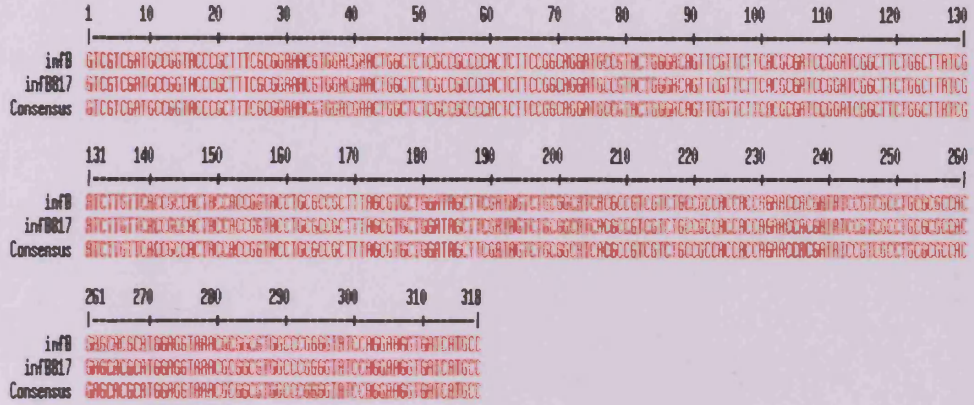

Figure B.35 Alignment of Pgi from *K. pneumoniae* AES817 sequence type as ST 511 with gene bank

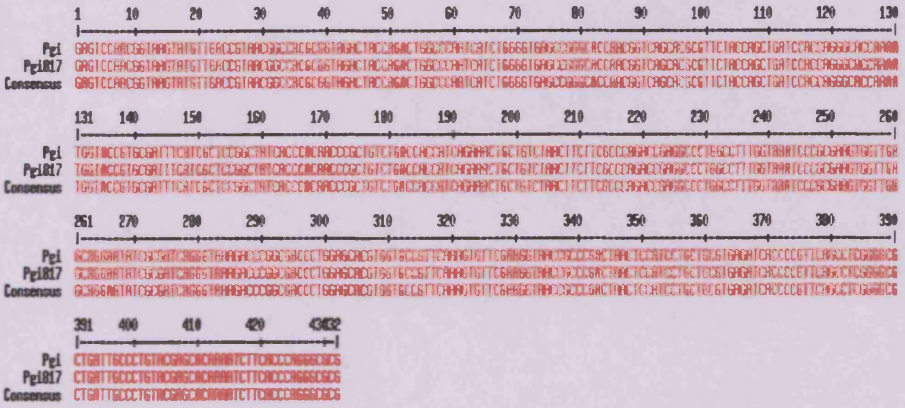

Figure B.36 Alignment of PhoE from *K. pneumoniae* AES817 sequence type as ST 511 with gene bank

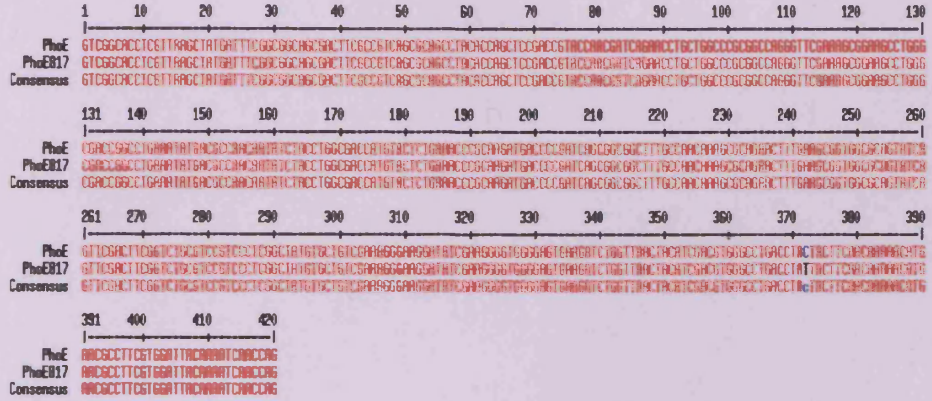

Figure B.37 Alignment of tnoB from *K. pneumoniae* AES817 sequence type as ST 511 with gene bank

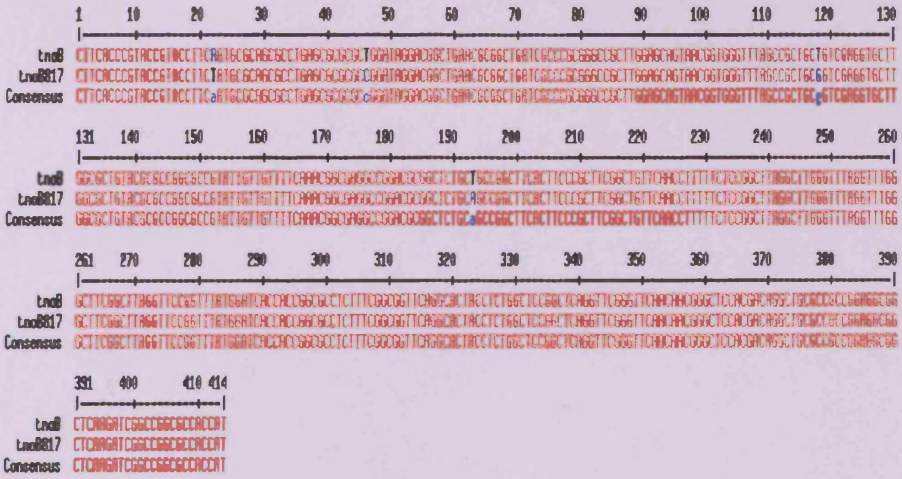

Figure B.38 Alignment of MDH from *K. pneumoniae* AES817 sequence type as ST 511 with gene bank

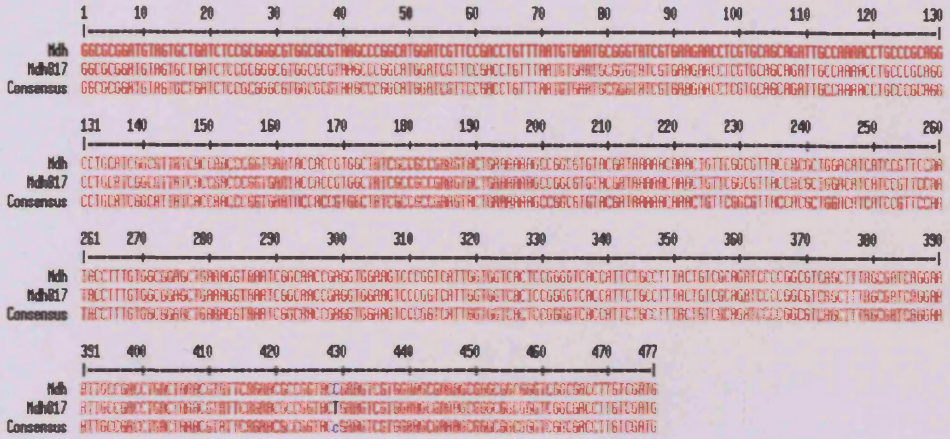

Figure B.39 Alignment of mdh from *K. pneumoniae* AES809 sequence type as ST 486 with gene bank

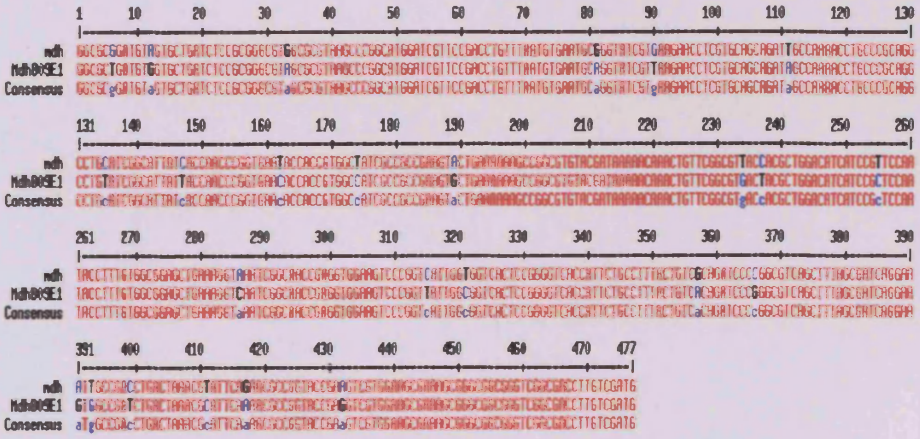

Figure B.40 Alignment of Pgi from *K. pneumoniae* AES809 sequence type as ST 486 with gene bank

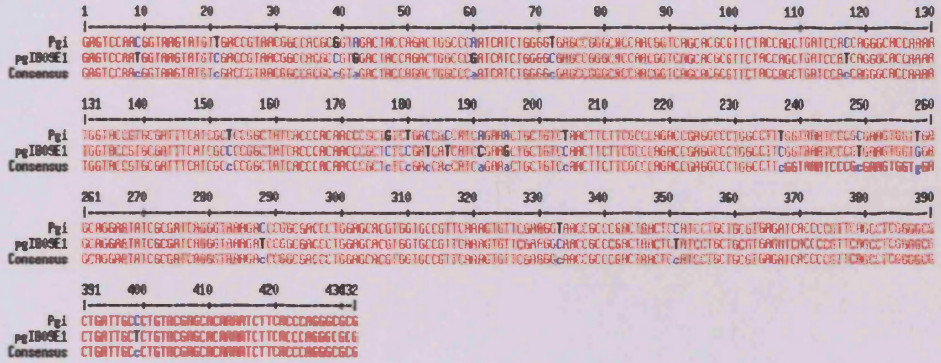

Figure B.41 Alignment of GapA from *K. pneumoniae* AES809 sequence type as ST 486 with gene bank

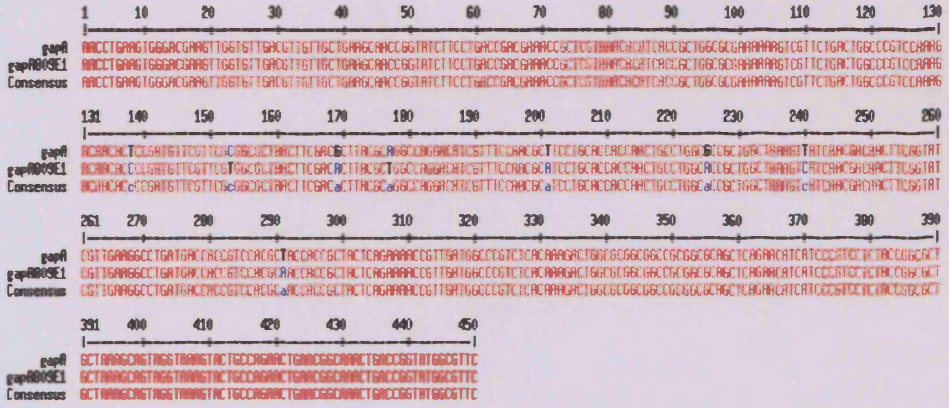

Figure B.42 Alignment of PhoE from *K. pneumoniae* AES809 sequence type as ST 486 with gene bank

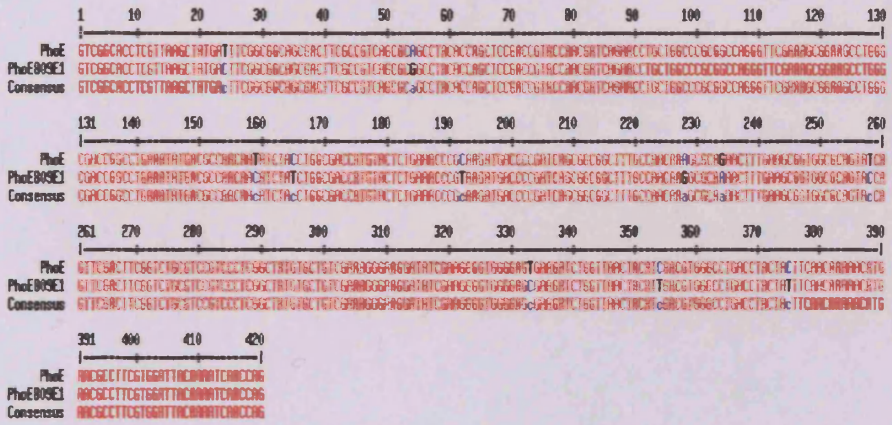

Figure B.43 Alignment of tnoB from *K. pneumoniae* AES809 sequence type as ST 486 with gene bank

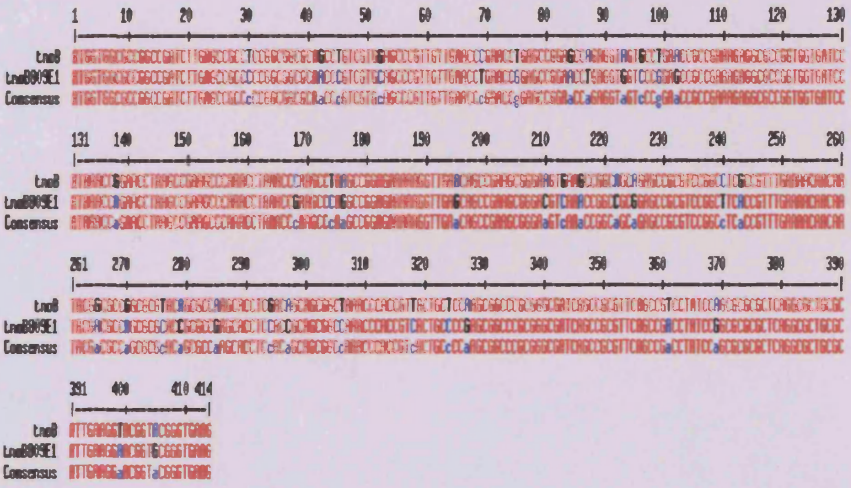

Figure B.44 Alignment of infB from *K. pneumoniae* AES809 sequence type as ST 486 with gene bank

Figure B.45 Alignment of RpoB from *K. pneumoniae* AES809 sequence type as ST 486 with gene bank

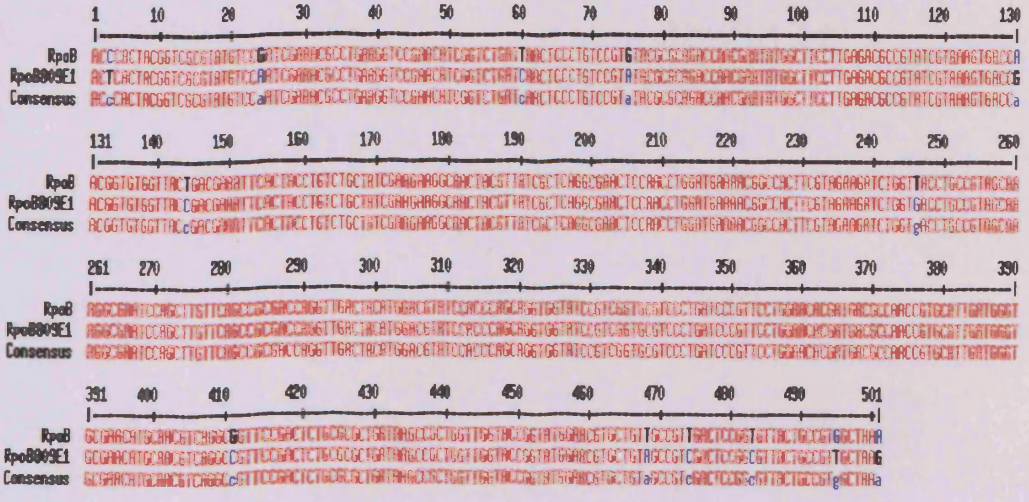

Figure B.46 Alignment of Pgi from *K. pneumoniae* AES808 sequence type as ST 509 with gene bank

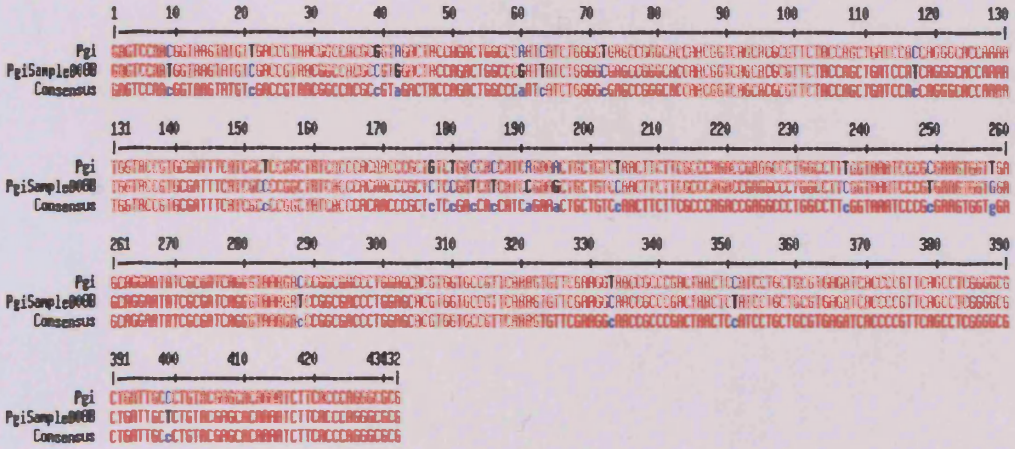

Figure B.47 Alignment of tnoB from *K. pneumoniae* AES808 sequence type as ST 509 with gene bank

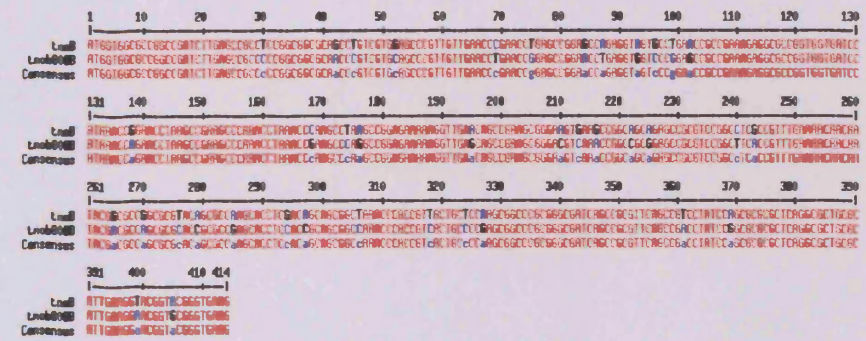

Figure B.48 Alignment of PhoE from *K. pneumoniae* AES808 sequence type as ST 509 with gene bank

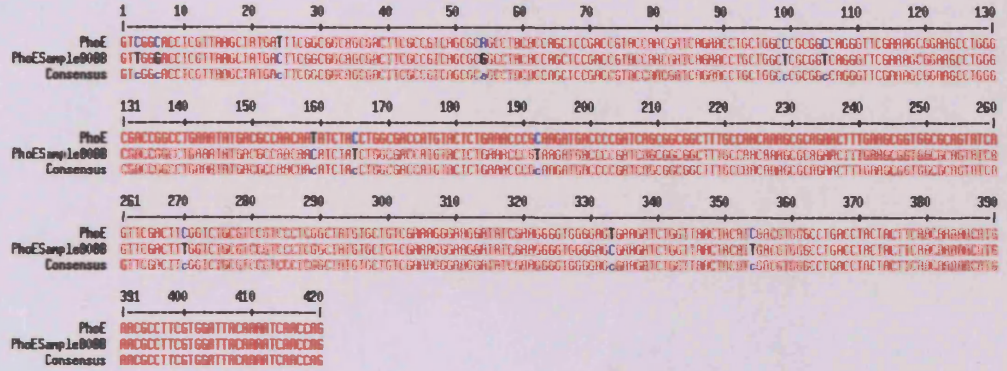

Figure B.49 Alignment of infB from *K. pneumoniae* AES808 sequence type as ST 509 with gene bank

Figure B.50 Alignment of RpoB from *K. pneumoniae* AES808 sequence type as ST 509 with gene bank

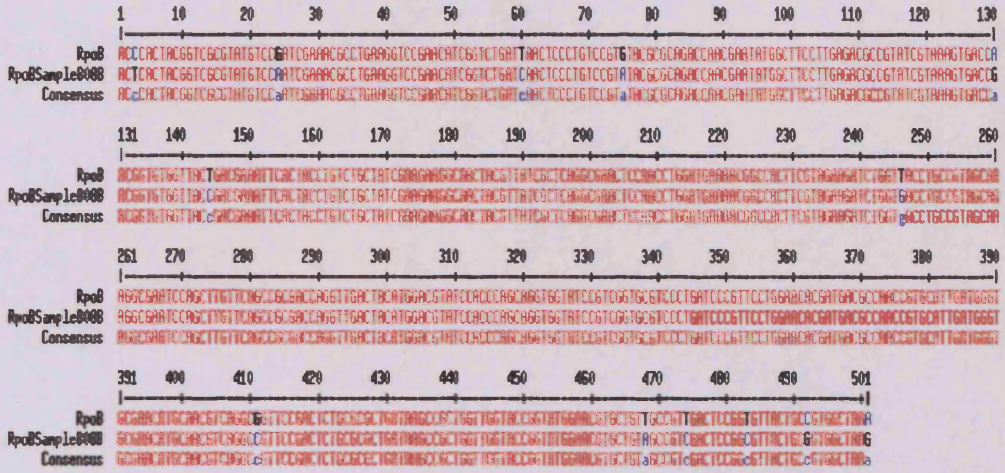

Figure B.51 Alignment of mdh from *K. pneumoniae* AES808 sequence type as ST 509 with gene bank

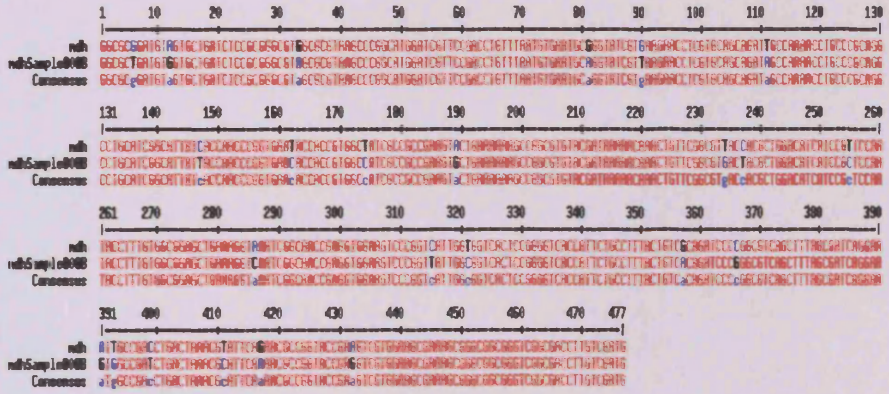

Figure B.52 Full sequence of AES81 integron

cttgaccgaacgcagcggtggtaacggcgcagtggcggttttcatggctt
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caagcgcgttacgccgtgggtcgatgtttgatgttatggagcagcaacga
tgttacgcagcagggcagtcgccctaaaacaaagttaggccgcatggaca
caacgcaggtcacattgatacacaaaattctagctgcggcagatgagcga
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ttgccgatggtgtttggtcgcatatcgcaacgcagtcgtttgatggcgca
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gattgatacagcgtggggtgcgaaaaacacagcggcacttctcgcggaga
ttgagaagcaaattggacttcctgtaacgcgtgcagtctccacgcacttt
catgacgaccgcgtcggcggcgttgatgtccttcgggcggctggggtggc
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agattcccacgcactctctagaaggactctcatcgagcggggacgcagtg
cgcttcggtccagtagaactcttctatcctggtgctgcgcattcgaccga
caacttagttgtgtacgtcccgtctgcgagtgtgctctatggtggttgtg
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tgctttaaccaacctaaagaaaataacggatcatgtcattgtttcaggtg
gtggggagatatacaaaagcctgatcgatcaagtagatacactacatata
tctacaatagacatcgagccggaaggtgatgtttactttcctgaaatccc
cagcaattttaggccagttttacccaagacttcgcctctaacataaatt
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agcgatggcggaagagtccgtaacaccgtacatcgcaatgctgggcgagg
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tggtgggaagatgaaactgatccaggagtgcgaggaatagaccagtctct
ggctgacccgacacagttgaacaaaggcctaggaacaaggcttgtccgcg
ctctcgttgaactactgttctcggacccaaccgtgacgaagattcagacc
gacccgactccgaacaaccatcgagccatacgctgctatgagaaggcagg
attcgtgcgggagaagatcatcaccacgcctgacgggccggcggtttaca
tggttcaaacacgacaagccttcgagagaaagcgcggtgttgcctaatgc
ctaactcagcgttcaagccgacgccgcttcgcggcgcggcttaattcagg

cgttagatgcactaagcacataattgctcacagccaaactatcaggtcaa
gtctgcttttattattttaagcgtgcataataagccctacacaaattgg
gagatatatcatgaaaggctgcgt

Figure B.53 Full sequence of AES83 integron

gaaccttgaccgaacgcagcggtggtaacggcgcagtggcggttttcatg
gcttgttatgactgtttttttggggtacagtctatgcctcgggcatccaa
gcagcaagcgcgttacgccgtgggtcgatgtttgatgttatggagcagca
acgatgttacgcagcagggcagtcgccctaaaacaaagttaggccgcatg
gacacaacgcaggtcacattgatacacaaaattctagctgcggcagatga
gcgaaatctgccgctctggatcggtgggggctgggcgatcgatgcacggc
tagggcgtgtaacacgcaagcacgatgatattgatctgacgtttcccggc
gagaggcgcggcgagctcgaggcaatagttgaaatgctcggcgggcgcgt
catggaggagttggactatggattcttagcggagatcggggatgagttac
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gccagtccgttgtaacagctgggaggcgatcatctgggattacttttact
atgccgatgaagtaccaccagtggactggcctacaaagcacatagagtcc
tacaggctcgcatgcacctcactcgggggcggaaaaggttgaggtcttgc
gtgccgctttcaggtcgcgatatgcggcctaacaattcgtccaagccgac
gccgcttcgcggcgcggcttaactcaggtgttaacctctgaggaagaatt
gtgaaactatcactaatggtagctatatcgaagaatggagttatcgggaa
tggccctgatattccatggagtgccaaaggtgaacagctcctgtttaaag
ctattacctataaccaatggctgttggttggacgcaagacttttgaatca
atgggagcattacccaaccgaaagtatgcggtcgtaacacgttcaagttt
tacatctgacaatgagaacgtattgatctttccatcaattaaagatgctt
taaccaacctaaagaaaataacggatcatgtcattgtttcaggtggtggg
gagatatacaaaagcctgatcgatcaagtagatacactacatatatctac
aatagacatcgagccggaaggtgatgtttactttcctgaaatccccagca
attttaggccagtttttacccaagacttcgcctctaacataaattatagt
taccaaatctggcaaaagggttaacaagtggcagcaacggattcgcaaac
ctgtcacgccttttgtaccaaaaagccgcgccaggtttgcgatccgctgt
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cgaccgactcttgatgaagtgctggaacactacctgcccagagcgatggc
ggaagagtccgtaacaccgtacatcgcaatgctgggcgaggaaccgatcg
gctatgctcagtcgtacgtcgcgctcggaagcggtgatggctggtgggaa
gatgaaactgatccaggagtgcgaggaatagaccagtctctggctgaccc
gacacagttgaacaaaggcctaggaacaaggcttgtccgcgctctcgttg
aactactgttctcggacccaaccgtgacgaagattcagaccgacccgact
ccgaacaaccatcgagccatacgctgctatgagaaggcaggattcgtgcg
ggagaagatcatcaccacgcctgacgggccggcggtttacatggttcaaa
cacgacaagccttcgagagaaagcgcggtgttgcctaacaactcattcaa
gccgacgccgcttcgcggcgcggcttaattcaggcgttagatgcactaag
cacataattgctcacagccaaactatcaggtcaagtctgcttttattatt
tttaagcgtgcataataagccctacacaaatngggagatatatcangaaa
gg

Figure B.54 Full sequence of AES135 integron

cgtagctgtaatgcaagtagcgtatgcgctcacgcaactggtccagaacc
ttgaccgaacgcagcggtggtaacggcgcagtggcggttttcatggcttg
ttatgactgttttttggggtacagtctatgcctcgggcatccaagcagc
aagcagcaagcgcgttacgccgtgggtcgatgtttgatgttatggagcag
caacgatgttacgcagcagggcagtcgccctaaaacaaagttaacccggg
accaaaattgtgaaagtatcattaatggctgcaagagcgagaaacggagt
gatcggttgcggtccacacataccctggtccgcgaaaggagagcagctac
tctttaaagccctgacgtacaaccagtggcttttggttggccgcaagacg
ttcgaatcaatgggggcgctccccaataggaaatacgcggtcgttactcg
ctcagcctggacggccaataatgacaacgtagtagtattcccgtcgatcg
aagaggccatgggcggtctagctaaactcaccggtcacgttatagtgtct
ggtggcggggagatttacagagaaacgttgcccatggcctctacgctcca
tgtatcgacgatcgacattgagccagaaggggatgttttcttcccgaata
ttcccaacttcttcgaagttgtttttgagcaacattttagttcaaacatt
aactattgctatcaaatttggaaaaagggttaacaaagctatgcaattga
cggcaaaaaagcttcgttcgccgcgctcactacgcttttaccgcaattg
atagcggcgttagatgcactaagcacataattgctcacagccaaactatc
aggtcaagtctgtttttattatttttaagcgtgcataataagccctacac
aaattgggagatatatcatgaaaggctggcttttcttgctatctcaata
gttggcgaagtaatcgcaacattcgcattaaaatctagcgagggctttac
taagcttgccccttccgccgctgtcataattggttatggcatcgcatttt
attttctttctctggttctgaaatccatccctgtcggtgttgcttatgca
gtctggtcgggactcggcgtcgtcataattacagccattgcctggttgct
tcatgggcaaaagcttgatgcgtggggctttgtaggtatggggctcatag
ttagtggtgtagtagttttaaacttgctttccaaagcaagtgcccactaa
taaactcagtcatctaacaagtcgttgcagcaccgctccagcacttcgtg
cctgcgctggacagtttttaagtcgcggctttatggttttgctgcgcaaa
agtattccataaaatcacaacttaaaaactgccgctgaactcggcgttga
acgacagctttcccaaaagctctacggctgctctgggtcgacaccggtaa
tcggatcgttgccgcactgaacagcgccccgttccaggtcgcctccattt
atgcggctgaaccgagggagagcagctttacgccgtctggccgcagttcg
cccttgggcgac

Appendix C

Table C.1 List of *E. coli* isolates collected from Tripoli and Benghazi

E. coli strain	Site of collection	Place of collection
AES11	Urine	Al-Jamhoryia hospital
AES35	Floor of toilet (ICU)	Al-Jamhoryia hospital
AES58	Blood	Al-Jamhoryia hospital
AES120	Wall of ICU	Al-Jamhoryia hospital
AES128	Urine	Al-Jamhoryia hospital
AES195	Urine	Al-Jamhoryia hospital
AES202	Urine	Al-Jamhoryia hospital
AES212	Swab from incubator	Benghazi Paediatric hospital
AES224	Floor of ICU	Benghazi Paediatric hospital
AES226	Urine	Benghazi Paediatric hospital
AES227	Wall of ICU	Benghazi Paediatric hospital
AES228	Floor of ICU	Benghazi Paediatric hospital
AES230	Bed side in ICU	Benghazi Paediatric hospital
AES231	Corner in ICU	Benghazi Paediatric hospital
AES232	Urine	Benghazi Paediatric hospital
AES237	Urine	Benghazi Paediatric hospital
AES239	Wall of ICU	Benghazi Paediatric hospital
AES240	Corridor of ICU	Benghazi Paediatric hospital
AES243	Urine	Benghazi Paediatric hospital
AES244	Urine	Benghazi Paediatric hospital
AES245	Urine	Benghazi Paediatric hospital
AES246	Urine	Benghazi Paediatric hospital
AES247	Floor of ICU	Benghazi Paediatric hospital
AES248	Blood	7^{th} of October hosital
AES262	Pus	7^{th} of October hospital
AES101	Floor of ICU	Al-Jamhoryia hospital
AES922	Urine	Al-Jalla hospital Tripoli
AES932	Urine	Maternity hospital Tripoli
AES937	Blood	Burn and plastic surgery Tripoli
AES938	Floor of ICU	Al-Jalla hospital Tripoli
AES941	Wall of ICU	Al-Jalla hospital Tripoli
AES944	Floor of toilet	Burn and plastic surgery Tripoli
AES962	Urine	Burn and plastic surgery Tripoli
AES964	Urine	Burn and plastic surgery Tripoli
AES966	Floor of ICU	Maternity hospital Tripoli
AES971	Bedside	Maternity hospital Tripoli
AES979	Urine	Maternity hospital Tripoli
AES1006	Blood	Burn and plastic surgery Tripoli
AES1037	Urine	Burn and plastic surgery Tripoli

Figure C.1 DNA sequence of *bla*$_{CTX-M-15}$ amplified from *E. coli* isolate AES226

NNNNNNNNNNNNNNANNNNNGANCNGAATCNGCGGCGCACGAT
CTTTTGGCCNGATCAC
CGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCC
CCCACAACCCAGGA
AGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCT
TTCATCCATGTCAC
CAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGA
TTCCGCANAGTTTG
CGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAAT
GGCGGTGTTTAACGT
CGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGG
GCGAACGCGGTGAC
GCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACG
TTATCGCTGTACTG
TAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACG
TGCTTTTCCGCAAT
CGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGAT
TTAACAGATTCGG
TTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTAC
TGGTGCTGCACAT
CGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCT
GTGTTAATCAATGC
CACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTT
GCTGTACGTCCGC
CGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTT
GCCGTCGCCATCNG
CGTGAACTGGCGCAGTGATTTTTTTAACCATGGGATTCCTTATTCTG
GAAGATACNAAAT
AACNACANNATGAATANNCCCCNANNNNCNCNNGNGNTTTTTNAT
TNNNNTTCANNNNCN
NNNNNNNNCAAAGNANNTNNNNNNGNNNCN

Figure C.2 Alignment of DNA sequence from figure C.1 with DNA sequences from gene bank

```
>  gb|HQ214045.1|  Enterobacter aerogenes strain K-307 plasmid
insertion sequence
ISEcp1 TnpA ISEcp1 (tnpA) gene, complete cds; and beta-lactamase
CTX-M-15 (blaCTX-M-15) and hypothetical protein genes,
complete cds
Length=2943

 Score = 1496 bits (810),  Expect = 0.0
 Identities = 821/830 (99%), Gaps = 1/830 (0%)
 Strand=Plus/Minus

Query  27
GAATCNGCGGCGCACGATCTTTTGGCCNGATCACCGCGATATCGTTGGTGGTGCCATAGC  86
              ||||| ||||||||||||||||||||
||||||||||||||||||||||||||||||||
Sbjct  2468
GAATCAGCGGCGCACGATCTTTTGGCCAGATCACCGCGATATCGTTGGTGGTGCCATAGC  2409

Query  87
CACCGCTGCCGGTTTTATCCCCCACAACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCG  146

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2408
CACCGCTGCCGGTTTTATCCCCCACAACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCG  2349

Query  147
CTGCACCGGTGGTATTGCCTTTCATCCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCA  206

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2348
CTGCACCGGTGGTATTGCCTTTCATCCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCA  2289

Query  207
ATGCTTTACCCAGCGTCAGATTCCGCANAGTTTGCGCCATTGCCCGAGGTGAAGTGGTAT  266
              |||||||||||||||||||||||||
||||||||||||||||||||||||||||||
Sbjct  2288
ATGCTTTACCCAGCGTCAGATTCCGCAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTAT  2229

Query  267
CACGCGGATCGCCCGGAATGGCGGTGTTTAACGTCGGCTCGGTACGGTCGAGACGGAACG  326

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2228
CACGCGGATCGCCCGGAATGGCGGTGTTTAACGTCGGCTCGGTACGGTCGAGACGGAACG  2169

Query  327
TTTCGTCTCCCAGCTGTCGGGCGAACGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAA  386

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2168
TTTCGTCTCCCAGCTGTCGGGCGAACGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAA  2109

Query  387
TCAGCTTATTCATCGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCA  446

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2108
TCAGCTTATTCATCGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCA  2049
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Query  447
GTGACATCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATT  506

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2048
GTGACATCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATT  1989

Query  507
TTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCG  566

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1988
TTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCG  1929

Query  567
CGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAA  626

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1928
CGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAA  1869

Query  627
GTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCC  686

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1868
GTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCC  1809

Query  687
GCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTC  746

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1808
GCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTC  1749

Query  747
CTAACAACAGCGTGACGGTTGCCGTCGCCATCNGCGTGAACTGGCGCAGTGAttttttttA  806
            ||||||||||||||||||||||||||||||||
|||||||||||||||||||||||| |
Sbjct  1748
CTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAACTGGCGCAGTGATTTTTT-A  1690

Query  807   ACCATGGGATTCCTTATTCTGGAAGATacnaaataacnacannatgaata  856
              ||||||||||||||||||||||||||||| ||||||| ||| |||||||
Sbjct  1689  ACCATGGGATTCCTTATTCTGGAAGATACGAAATAACAACAACATGAATA  1640


>  gb|HQ214044.1|  Enterobacter cloacae strain K-221 plasmid
IncF::FIB insertion
sequence ISEcp1 TnpA ISEcp1 (tnpA) gene, complete cds; and
beta-lactamase CTX-M-15 (blaCTX-M-15) and hypothetical protein
genes, complete cds
Length=2943

 Score = 1496 bits (810),  Expect = 0.0
 Identities = 821/830 (99%), Gaps = 1/830 (0%)
 Strand=Plus/Minus

Query  27
GAATCNGCGGCGCACGATCTTTTGGCCNGATCACCGCGATATCGTTGGTGGTGCCATAGC  86
            ||||| ||||||||||||||||||||
||||||||||||||||||||||||||||||||
Sbjct  2468
GAATCAGCGGCGCACGATCTTTTGGCCAGATCACCGCGATATCGTTGGTGGTGCCATAGC  2409
```

```
Query  87
CACCGCTGCCGGTTTTATCCCCCACAACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCG  146

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2408
CACCGCTGCCGGTTTTATCCCCCACAACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCG  2349

Query  147
CTGCACCGGTGGTATTGCCTTTCATCCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCA  206

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2348
CTGCACCGGTGGTATTGCCTTTCATCCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCA  2289

Query  207
ATGCTTTACCCAGCGTCAGATTCCGCANAGTTTGCGCCATTGCCCGAGGTGAAGTGGTAT  266
               ||||||||||||||||||||||||||
||||||||||||||||||||||||||||||||
Sbjct  2288
ATGCTTTACCCAGCGTCAGATTCCGCAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTAT  2229

Query  267
CACGCGGATCGCCCGGAATGGCGGTGTTTAACGTCGGCTCGGTACGGTCGAGACGGAACG  326

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2228
CACGCGGATCGCCCGGAATGGCGGTGTTTAACGTCGGCTCGGTACGGTCGAGACGGAACG  2169

Query  327
TTTCGTCTCCCAGCTGTCGGGCGAACGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAA  386

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2168
TTTCGTCTCCCAGCTGTCGGGCGAACGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAA  2109

Query  387
TCAGCTTATTCATCGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCA  446

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2108
TCAGCTTATTCATCGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCA  2049

Query  447
GTGACATCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATT  506

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2048
GTGACATCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATT  1989

Query  507
TTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCG  566

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1988
TTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCG  1929

Query  567
CGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAA  626

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1928
CGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAA  1869

Query  627
GTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCC  686

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```
Sbjct  1868
GTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCC  1809

Query  687
GCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTC  746

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1808
GCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTC  1749

Query  747
CTAACAACAGCGTGACGGTTGCCGTCGCCATCNGCGTGAACTGGCGCAGTGAtttttttA  806
              ||||||||||||||||||||||||||||||||
||||||||||||||||||||||||| |
Sbjct  1748
CTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAACTGGCGCAGTGATTTTTT-A  1690

Query  807   ACCATGGGATTCCTTATTCTGGAAGATacnaaataacnacannatgaata  856
              |||||||||||||||||||||||||||| ||||||| |||  |||||||
```

Figure C.3 DNA sequence of *bla*$_{CTX-M-15}$ amplified from *E. coli* isolate AES228

GCGGCGCACGATCTTTTGGCCNGATCAC
CGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTA
TCCCCCACAACCCAGGA
AGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTG
CCTTTCATCCATGTCAC
CAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTC
AGATTCCGCANAGTTTG
CGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGA
ATGGCGGTGTTTAACGT
CGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGT
CGGGCGAACGCGGTGAC
GCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCC
ACGTTATCGCTGTACTG
TAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTG
ACGTGCTTTTCCGCAAT
CGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCT
GATTTAACAGATTCGG
TTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTT
TACTGGTGCTGCACAT
CGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCT
GCTGTGTTAATCAATGC
CACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGT
TTTTGCTGTACGTCCGC
CGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACG
GTTGCCGTCGCCATCNG
CGTGAACTGGCGCAGTGATTTTTTTAACCATGGGATTCCTTATT
CTGGAAGATACNAAAT
AACNACANNATGAATANNCCCCNANNNNCNCNNGNGNTTTTT

Figure C.4 Alignment of DNA sequence from figure C.3 with DNA sequences from gene bank

```
gb|JN788267.1| Acinetobacter baumannii strain H1
hydroxyisourate hydrolase gene,
complete cds; disrupted pyrimidine utilization transporter
gene, partial sequence; insertion sequence ISEcp1 transposase
(tnpA) gene, complete cds; CTX-M15 (blaCTX-M15) gene,
complete cds; disrupted orf477 gene, partial sequence;
transposon
Tn3 tnpA gene, partial sequence; and hypothetical protein
gene, complete cds
Length=5224

 Score = 1489 bits (806),  Expect = 0.0
 Identities = 816/824 (99%), Gaps = 1/824 (0%)
 Strand=Plus/Minus
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```
Query  1
GCGGCGCACGATCTTTTGGCCNGATCACCGCGATATCGTTGGTGGTGCCATAGCCACCGC
60
               ||||||||||||||||||||||
||||||||||||||||||||||||||||||||||||||
Sbjct  3516
GCGGCGCACGATCTTTTGGCCAGATCACCGCGATATCGTTGGTGGTGCCATAGCCACCGC
3457

Query  61
TGCCGGTTTTATCCCCCACAACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCGCTGCAC
120

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Sbjct  3456
TGCCGGTTTTATCCCCCACAACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCGCTGCAC
3397

Query  121
CGGTGGTATTGCCTTTCATCCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCAATGCTT
180

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Sbjct  3396
CGGTGGTATTGCCTTTCATCCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCAATGCTT
3337

Query  181
TACCCAGCGTCAGATTCCGCANAGTTTGCGCCATTGCCCGAGGTGAAGTGGTATCACGCG
240
               ||||||||||||||||||||||
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Sbjct  3336
TACCCAGCGTCAGATTCCGCAGAGTTTGCGCCATTGCCCGAGGTGAAGTGGTATCACGCG
3277

Query  241
GATCGCCCGGAATGGCGGTGTTTAACGTCGGCTCGGTACGGTCGAGACGGAACGTTTCGT
300

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Sbjct  3276
GATCGCCCGGAATGGCGGTGTTTAACGTCGGCTCGGTACGGTCGAGACGGAACGTTTCGT
3217

Query  301
CTCCCAGCTGTCGGGCGAACGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAATCAGCT
360

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Sbjct  3216
CTCCCAGCTGTCGGGCGAACGCGGTGACGCTAGCCGGGCCGCCAACGTGAGCAATCAGCT
3157

Query  361
TATTCATCGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACA
420

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Sbjct  3156
TATTCATCGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCAGTGACA
3097

Query  421
TCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGA
480

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Sbjct  3096
TCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGA
3037

Query  481
TCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCG
540

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Sbjct  3036
TCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCG
2977

Query  541
CGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTT
600

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Sbjct  2976
CGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTT
2917

Query  601
GCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTA
660

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Sbjct  2916
GCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCCGCTCTA
2857

Query  661
ATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACA
720

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Sbjct  2856
ATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTCCTAACA
2797

Query  721
ACAGCGTGACGGTTGCCGTCGCCATCNGCGTGAACTGGCGCAGTGAttttttttAACCATG
780
              ||||||||||||||||||||||||||
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Sbjct  2796
ACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAACTGGCGCAGTGATTTTTT-AACCATG
2738

Query  781   GGATTCCTTATTCTGGAAGATACNAAATAACNACANNATGAATA  824
              ||||||||||||||||||||||| ||||||| |||  |||||||
Sbjct  2737  GGATTCCTTATTCTGGAAGATACGAAATAACAACAACATGAATA  2694
```

Figure C.5 DNA sequence of *bla*$_{CTX-M-15}$ amplified from *E. coli* isolate AES232

CGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCC
CCCACAACCCAGGA
AGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCT
TTCATCCATGTCAC
CAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGA
TTCCGCANAGTTTG
CGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAAT
GGCGGTGTTTAACGT
CGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGG
GCGAACGCGGTGAC
GCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACG
TTATCGCTGTACTG
TAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACG
TGCTTTTCCGCAAT
CGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGAT
TTAACAGATTCGG
TTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTAC
TGGTGCTGCACAT
CGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCT
GTGTTAATCAATGC
CACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTT
GCTGTACGTCCGC
CGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTT
GCCGTCGCCATCNG
CGTGAACTGGCGCAGTGATTTTTTTAACCATGGGATTCCTTATTCTG
GAAGATACNAAAT
AACNACANNATGAATANNCCCCNANNNNCNCNNGNGNTTTTTNNN
NNN

Figure C.6 Alignment of DNA sequence from figure C.5 with DNA sequences from gene bank

emb|FR828676.1| Escherichia coli plasmid pCTX913 tnpA gene,
blaCTX-M-15 gene
and delta tnpA gene (partial), isolate 913
Length=2656

Score = 1441 bits (780), Expect = 0.0
Identities = 789/796 (99%), Gaps = 1/796 (0%)
Strand=Plus/Minus

Query 1
CGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCCCCCACAACCCAGGA
60

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Sbjct  1674
CGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTTTATCCCCCACAACCCAGGA
1615

Query  61
AGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCATCCATGTCAC
120

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Sbjct  1614
AGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCATCCATGTCAC
1555

Query  121
CAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGATTCCGCANAGTTTG
180

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Sbjct  1554
CAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGATTCCGCAGAGTTTG
1495

Query  181
CGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAATGGCGGTGTTTAACGT
240

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Sbjct  1494
CGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAATGGCGGTGTTTAACGT
1435

Query  241
CGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGGGCGAACGCGGTGAC
300

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Sbjct  1434
CGGCTCGGTACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGGGCGAACGCGGTGAC
1375

Query  301
GCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACGTTATCGCTGTACTG
360

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Sbjct  1374
GCTAGCCGGGCCGCCAACGTGAGCAATCAGCTTATTCATCGCCACGTTATCGCTGTACTG
1315

Query  361
TAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTTTTCCGCAAT
420

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Sbjct  1314
TAGCGCGGCCGCGCTAAGCTCAGCCAGTGACATCGTCCCATTGACGTGCTTTTCCGCAAT
1255

Query  421
CGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACAGATTCGG
480
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1254
CGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACAGATTCGG
1195

Query  481
TTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGCTGCACAT
540

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Sbjct  1194
TTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGCTGCACAT
1135

Query  541
CGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAATCAATGC
600

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Sbjct  1134
CGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAATCAATGC
1075

Query  601
CACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGC
660

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Sbjct  1074
CACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGC
1015

Query  661
CGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCGCCATCNG
720

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Sbjct  1014
CGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCGCCATCAG
955

Query  721
CGTGAACTGGCGCAGTGAttttttttAACCATGGGATTCCTTATTCTGGAAGATacnaaat
780
              |||||||||||||||||||||||||
|||||||||||||||||||||||||||||| ||||
Sbjct  954   CGTGAACTGGCGCAGTGATTTTTT-
AACCATGGGATTCCTTATTCTGGAAGATACGAAAT  896

Query  781   aacnacannatgaata  796
              ||| |||  |||||||
Sbjct  895   AACAACAACATGAATA  880
```

Figure C.7 DNA sequence of *bla*$_{CTX-M-3}$ amplified from *E. coli* isolate AES228 amplified by CTX-M-F

NNNNNNNNNCNNNNGCNGTTGTTAGGAGTGTGCCGCTGTATGCGC
AAACGGCGGACGTAC
AGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGG
GTGTGGCATTGATTA
ACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTT
TGCGATGTGCAGCA
CCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAA
GCGAACCGAATCTGT
TAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAA
TCCGATTGCGGAAA
AGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCG
CGCTACAGTACAGCG
ATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGC
TAGCGTCACCGCGT
TCGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGA
GCCGACGTTAAACA
CCGCCATTCCGGGCGATCCGCGTGATACCNCTTCACCTCNGGCAAT
GGCGCANANTCTGC
GGAATCTGACGCTGGGNAANGNNTNGGGCGACNNCNNACNGGCGC
NNCTGGTGANN

Figure C.8 Alignment of DNA sequence from figure C.7 with DNA sequence from gene bank

```
>  gb|HQ214052.1|  Enterobacter cloacae strain S-440 plasmid
IncF::FIB insertion
sequence ISEcp1, partial sequence; and beta-lactamase CTX-M-3
(blaCTX-M-3) and hypothetical protein genes, complete cds
Length=1498

 Score = 1088 bits (589),  Expect = 0.0
 Identities = 600/611 (98%), Gaps = 0/611 (0%)
 Strand=Plus/Minus

Query  17
AGCTCNGCCNGTGACNTCGTCCCNTTGACGTGCTTTTCCGCAATCGGATTATAGTTAACA  76
              ||||| ||| ||||| |||||||
||||||||||||||||||||||||||||||||||||
Sbjct  613
AGCTCAGCCAGTGACATCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACA  554

Query  77
AGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTC  136

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Sbjct  553
AGGTCAGATTTTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTC  494

Query  137
TTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCA  196

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Sbjct  493
TTCAGCACCGCGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCA  434

Query  197
GCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCT  256

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Sbjct  433
GCACGATAAAGTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCT  374

Query  257
CCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGC  316

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Sbjct  373
CCCGACTGCCGCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGC  314

Query  317
GGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAACTGGCGCAGT  376

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Sbjct  313
GGCACACTTCCTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAACTGGCGCAGT  254

Query  377
GATTTTTTAACCATGGGATTCCTTATTCTGGAAGAGACGAAATAACAACAACATGAATAG  436

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Sbjct  253
GATTTTTTAACCATGGGATTCCTTATTCTGGAAGAGACGAAATAACAACAACATGAATAG  194

Query  437
TCAATATTTTACCTGAAGCGAGCCACAACGCGTCCGATTTTATGCTTCCGAAAGGCAAAT  496

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Sbjct  193
TCAATATTTTACCTGAAGCGAGCCACAACGCGTCCGATTTTATGCTTCCGAAAGGCAAAT  134

Query  497
ACNGACGTCGCCAGAATGAAACCTAAATTCCACGTGTGTTTTTTATTANCTnnnanaaTC  556
             || |||||||||||||||||||||||||||||||||||||||||||| ||    |
||||
Sbjct  133
ACGGACGTCGCCAGAATGAAACCTAAATTCCACGTGTGTTTTTTATTAGCTTCAAAAATC  74

Query  557
ACTATTTCACGAAGAATTTAGACTGCTTCTCACACATTGTAACATTATTTACAACCNCCT  616
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
|||
Sbjct  73
ACTATTTCACGAAGAATTTAGACTGCTTCTCACACATTGTAACATTATTTACAACCACCT  14

Query  617  TTCAATCATTT  627
             ||||||||||||
Sbjct  13   TTCAATCATTT  3
```

Figure C.9 DNA sequence of *bla*$_{\text{CTX-M-3}}$ amplified from *E. coli* isolate AES226 amplified by CTX-M-F

CNGTGACATCGTCCCNTTGACGTGCTTTTCCGCAAT
CGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGAT
TTAACAGATTCGG
TTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTAC
TGGTGCTGCACAT
CGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCT
GTGTTAATCAATGC
CACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTT
GCTGTACGTCCGC
CGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTT
GCCGTCGCCATCAG
CGTGAACTGGCGCAGTGATTTTTTAACCATGGGATTCCTTATTCTGG
AAGANACGAAATA
ACAACAACATGAATAGTCNATATTTTACCTGAGGGGAGNCAANNN
NCNTCNNANTTNANG
CTTCCGAAAGGAAAATACAGAGNTCNNCANAANGAAANNNAATAT
NNACNNGTGNNTTTN
ANNNNNNTTNTAAAATCACTATTTCACGAAGAATTTAGACTGCTTC
TCACACATTGTAAC
ATTATTTACAACCACCTTTCNNNNNNNNNNNNNNNNNNGNNNNG
NNNNNCNNANNNNNN
CNNNNNNNNCNNCCNTTGTNNTCNTTTNCGCANNNGNNTGACNCA
CTCNCNNANTANNNNANNNNNNNCNTTTCCTTTTNTNNNNNNCGN
GNNCGNNN

NNNNCN

Figure C.10 Alignment of DNA sequence from figure C.9 with DNA sequence from gene bank

```
Escherichia coli strain S-741 plasmid IncL/M insertion sequence
ISEcp1, partial sequence; beta-lactamase CTX-M-3 (blaCTX-M-3)
and hypothetical protein genes, complete cds; and MucA
(mucA) gene, partial cds
Length=2028

 Score =  907 bits (491),  Expect = 0.0
 Identities = 552/597 (92%), Gaps = 7/597 (1%)
 Strand=Plus/Minus

Query  3
GTGACATCGTCCCNTTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATT
62
             ||||||||||||
||||||||||||||||||||||||||||||||||||||||||||
Sbjct  603
GTGACATCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATT
544

Query  63
TTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCG
122

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Sbjct  543
TTTTGATCTCAACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCG
484

Query  123
CGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAA
182

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Sbjct  483
CGGCCGCGGCCATCACTTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAA
424

Query  183
GTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCC
242

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Sbjct  423
GTATTTGCGAATTATCTGCTGTGTTAATCAATGCCACACCCAGTCTGCCTCCCGACTGCC
364

Query  243
GCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTC
302

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Sbjct  363
GCTCTAATTCGGCAAGTTTTTGCTGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTC
304

Query  303
CTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAACTGGCGCAGTGATTTTTTAA
362

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Sbjct  303
CTAACAACAGCGTGACGGTTGCCGTCGCCATCAGCGTGAACTGGCGCAGTGATTTTTTAA
244

Query  363
CCATGGGATTCCTTATTCTGGAAGANACGAAATAACAACAACATGAATAGTCNATATTTT
422
            |||||||||||||||||||||||||
||||||||||||||||||||||||| |||||||
Sbjct  243
CCATGGGATTCCTTATTCTGGAAGAGACGAAATAACAACAACATGAATAGTCAATATTTT
184

Query  423  ACCTGAGGGGAGnca-
annnncntcnnanttnangcttccgaaaggaaaatacaga-gnt  480
            |||||| | ||| || |    | ||  | || | ||||||||||||
|||||| || | |
Sbjct  183  ACCTGAAGCGAGCCACAACG-
CGTCCGATTTTATGCTTCCGAAAGGCAAATACGGACG-T  126

Query  481  cnncanaangaaannnaatatnn-
acnngtgnntttnannnnnnttntaaaaTCACTATT  539
            |  || || ||||   || ||   ||  |||  ||| |      ||
||||||||||||
Sbjct  125  CGCCAGAATGAAACCTAA-ATTCCACGTGTGTTTTTTATTAGC-
TTCAAAAATCACTATT  68

Query  540
TCACGAAGAATTTAGACTGCTTCTCACACATTGTAACATTATTTACAACCACCTTTC  596

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Sbjct  67
TCACGAAGAATTTAGACTGCTTCTCACACATTGTAACATTATTTACAACCACCTTTC  11
```

Figure C.11 DNA sequence of *bla*$_{CTX-M-3}$ amplified from *E. coli* isolate AES232 amplified by CTX-M-F

NNNNNNNNNNNGCGCNANCTNNGCCNGTGACNTCGTCCCNTTGAC
GTGCTTTTCCGCAAT
CGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCGCTGAT
TTAACAGATTCGG
TTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCACTTTAC
TGGTGCTGCACAT
CGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATCTGCT
GTGTTAATCAATGC
CACACCCAGTCTGCCTCCCGACTGCCGCTCTAATTCGGCAAGTTTTT
GCTGTACGTCCGC
CGTTTGCGCATACAGCGGCACACTTCCTAACAACAGCGTGACGGTT
GCCGTCGCCATCAG
CGTGAACTGGCGCAGTGATTTTTTAACCATGGGATTCCTTATTCTGG
AAGANACGAAATA
ACAACAACATGAATAGTCNATATTTTACNTGANNGNNGNCNNNNN
NCNNCNNANTTNATG
CTTCCNAAAGGAAAATANANNNNTNNNCNGAANGNNNNNNANNN
NNNACNNGNGNNNTTA
NNNNNNNTTTNAAAATCACTATTTCACGAAGAATTTAGACTGCTTC
TCACACATTGNAAC
NNNNNTTNNNAACCNCCTTTNNNNNNNNTTNNNNNNANNNNGGGA
NCNNGNNCNNNAANN
NNNNNCNNNGNNNTCNTNCCNNTNNNNTGCTTTTCNGCAATCNGA
TTATANTTTANNNGG
NCNNANTTNTTGANCNCNNTCNCNNNNNNANNNAATTNNGNNTCN
NTTNCTTTTNNNTCN
NNNCNNNNNCCNNNNNCNTNCTTTNNNGNNNCTNNNCATNCAANN
NNCTCNTCNNNNCAT
NANNNNNNNNNANTNTCNGCNGNNNTTAANNANGNNNNNNNNNG
NNNCCNANGNNNNNNA
ANTNNNGCANNNTTTTTNNNNNNNNNNNGNNNNNN

Figure C.12 Alignment of DNA sequence from figure C.11 with DNA sequence from gene bank

gb|GQ292713.1| Klebsiella pneumoniae strain S-334 plasmid IncL/M insertion sequence
IS26 transposase tnpA IS26 (tnpA) gene, complete cds; insertion sequence ISEcp1, complete sequence; beta-lactamase CTX-M-3 (blaCTX-M-3) gene, complete cds; MucA (mucA) gene, partial cds; and unknown gene
Length=3260

Score = 747 bits (404), Expect = 0.0
Identities = 407/410 (99%), Gaps = 0/410 (0%)

```
 Strand=Plus/Minus

Query  1
GACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCG
60

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1803
GACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTCAACTCG
1744

Query  61
CTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCAC
120

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Sbjct  1743
CTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCGCGGCCATCAC
1684

Query  121
TTTACTGGTGCTGCACATCGCAAAGCGCTCATCAGCACGATAAAGTATTTGCGAATTATC
180

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1683
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               ||||||||| |||||||||||||||||||||||| ||||||||| |||
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1394
```

Appendix D

TABLE D.1 Ceftazidime resistant Gram-negative bacteria isolated from Hospital environmental swabs.

Swab	Bacterial isolate	Location
301	*Achromobacter* sp	Tripoli central hospital
302	*Pseudomonas putida*	Gergarish
303	*Aeromonas caviae*	1st of September
304	*Achromobacter* sp.	Andalus
305	*Acinetobacter baumannii*	Gergarish
306	*Stenotrophomonas maltophilia*	Omar Mokhtar
307	*Pseudomonas pseudoalcaligenes*	Omar Mokhtar
308	*Stenotrophomonas maltophilia*	Seraj
309	*Achromobacter* sp.	Seraj area
310	*Achromobacter* sp	Siahia
311	*Pantoea agglomerans*	Seraj
312	*Pseudomonas aeruginosa*	Siahia
313	*Achromobacter* sp	Omar Mokhtar
314	*Tatumella ptyseos*	Siahia
315	*Pseudomonas putida*	Seraj
316	*Pseudomonas putida*	Omar Mokhtar
317	*Achromobacter* sp	Omar Mokhtar
318	*Burkholderia cepacia*/Ralstonia pickettii	Seraj
319	*Achromobacter* sp	Seraj
320	*Pseudomonas putida*	Gergarish
321	*Pseudomonas putida*	Seraj
322	*Stenotrophomonas maltophilia*	Seraj
323	*Tatumella ptyseos*	Gergarish
324	*Achromobacter* sp	Seraj
325	*Enterobacter cloacae*	Omar Mokhtar
326	*Citrobacter freundii*	Seraj
327	*Tatumella ptyseos*	Seraj

328	*Pantoea agglomerans*	Jamahiyria
329	*Achromobacter* sp	Jamahiyria
330	*Achromobacter* sp	Jamahiyria
331	*Ochrobactrum anthropi*	Jamahiyria
332	*P. aeruginosa*	Serah
333	*Achromobacter* sp	Seraj
334	*Achromobacter* sp	Seraj
335	Acinetobacter baumannii	Gergarish
336	*Leclercia adecarboxyalata*	Seraj
337	*Stenotrophomonas maltophilia*	Akhadra
338	*Enterobacter cloacae*	Alkhadra

Figure D.1 Hydrolysis of antibiotic meropenem by TMB-1

	Vo #1	Vo	Vo #3 [E]: 100nM
VMAX	6.102		26.42
KM	355.2		75.11

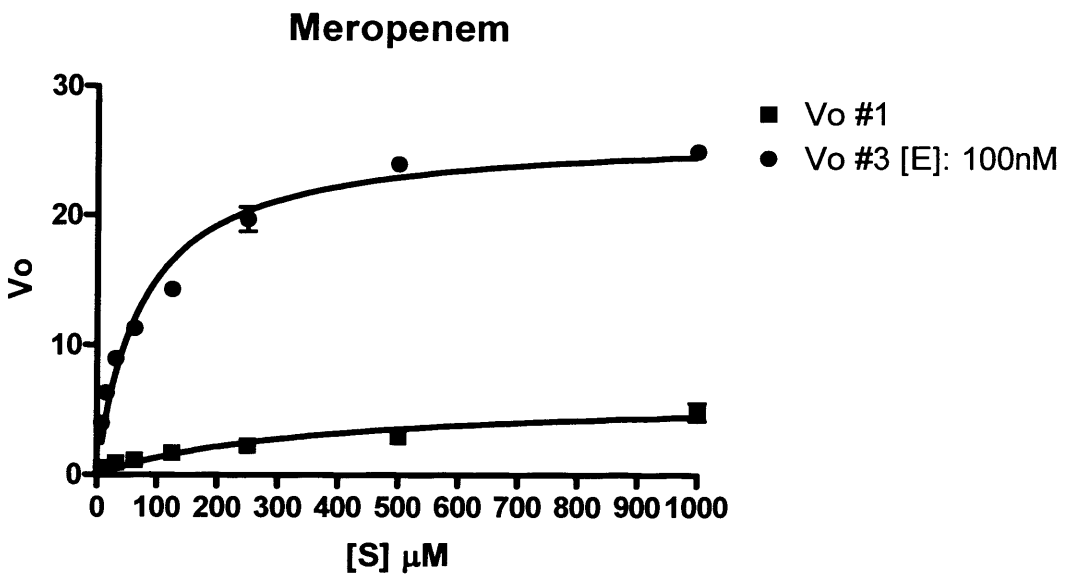

Figure D.2 Hydrolysis of antibiotic Ertapenem by TMB-1

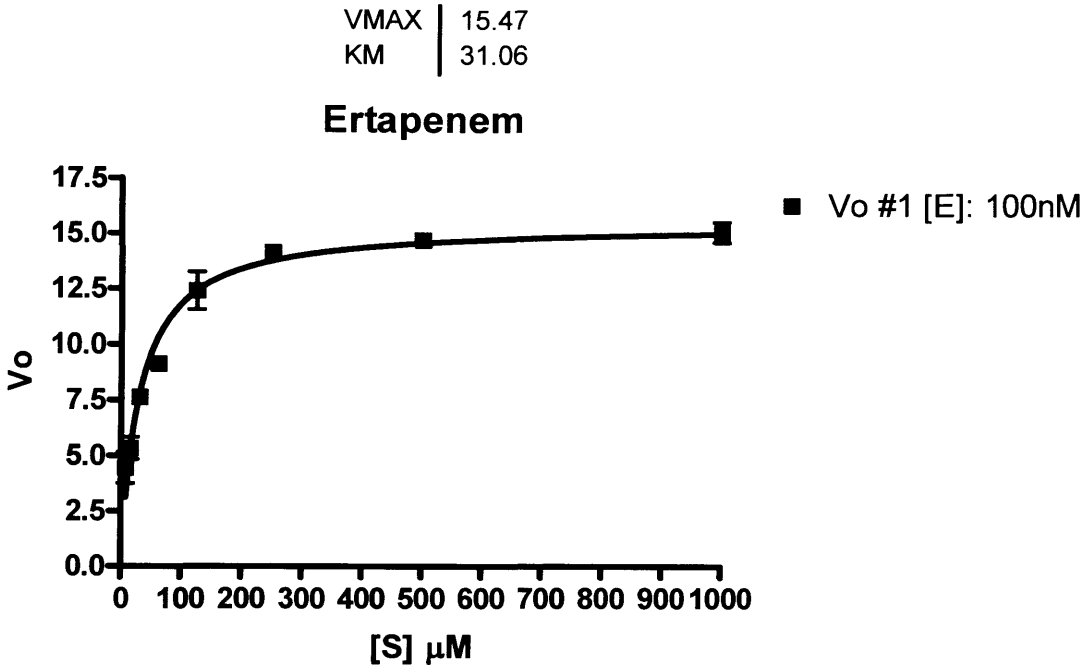

Figure D.3 Hydrolysis of antibiotic ceftazidime by TMB-1

	Vo #1 [E]: 1 μM	Vo #3 [E]: 100nM	Vo #3 [E]: 1 μM
VMAX	1.927	2.632	2.577
KM	18.89	187.6	91.81

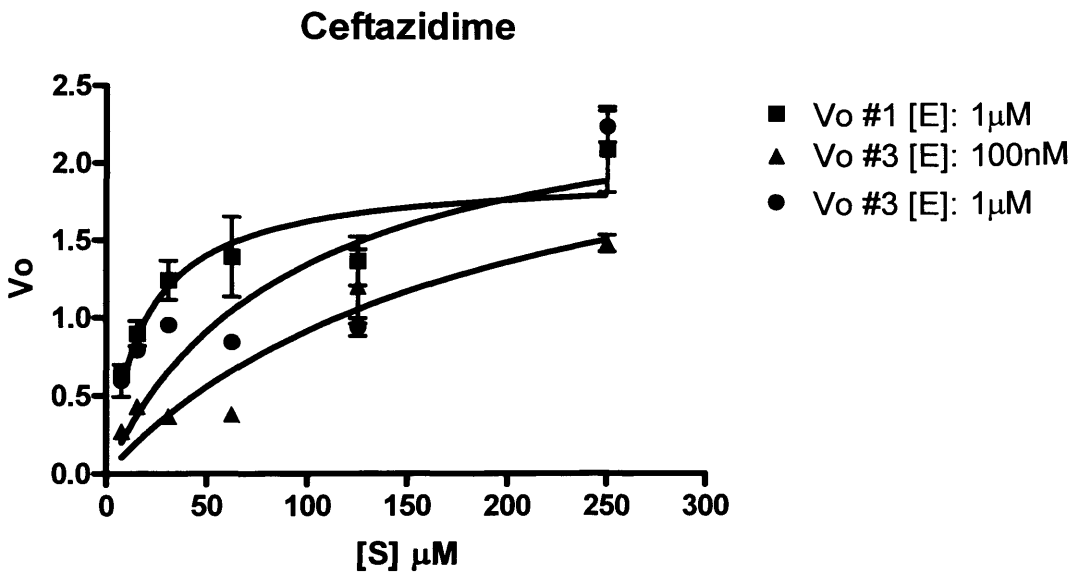

Figure D.4 Hydrolysis of antibiotic ampicilin by TMB-1

	Vo #1 [E]: 100nM	Vo #2 [E]: 100nM
VMAX	4.490	5.822
KM	11.82	27.37

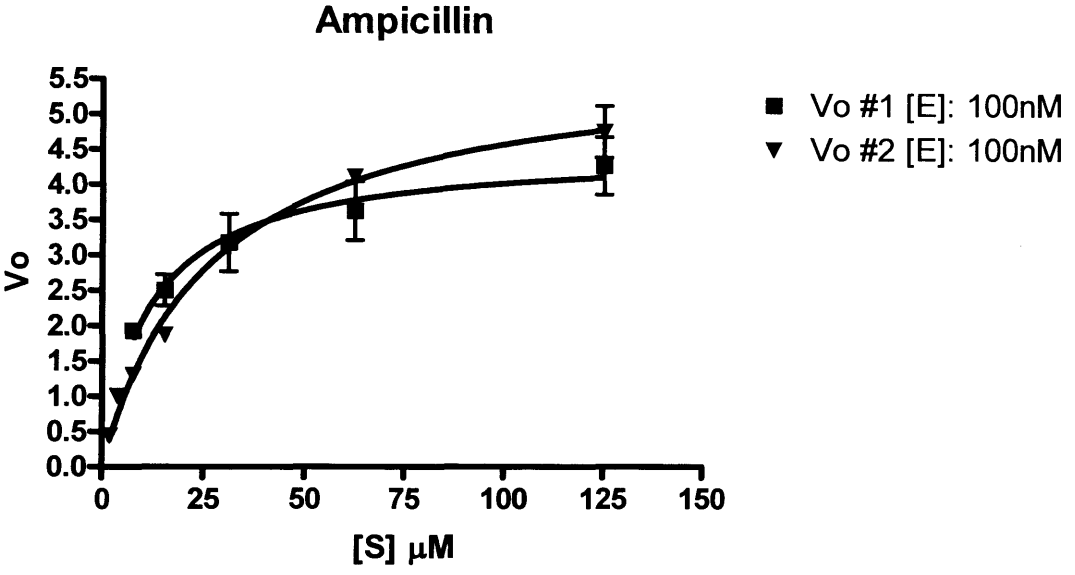

Figure D.5 Hydrolysis of antibiotic imipenem by TMB-1

	Vo #1 [E]: 10nM	Vo #2 [E]: 10nM	Vo #3 [E]:100nM
VMAX	21.07	11.60	35.30
KM	1909	614.0	200.8

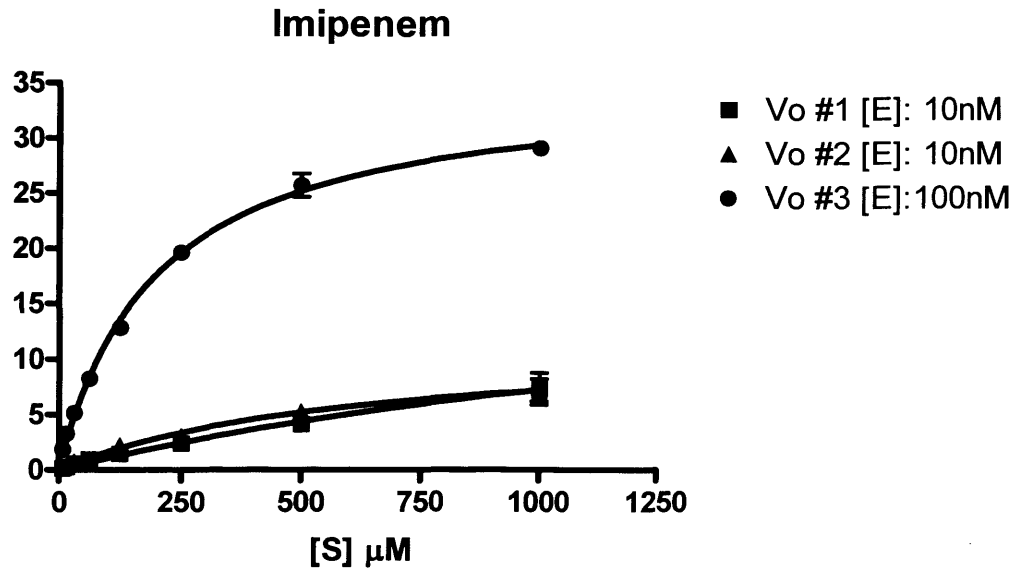

Figure D.6 Hydrolysis of antibiotic cefoxitin by TMB-1

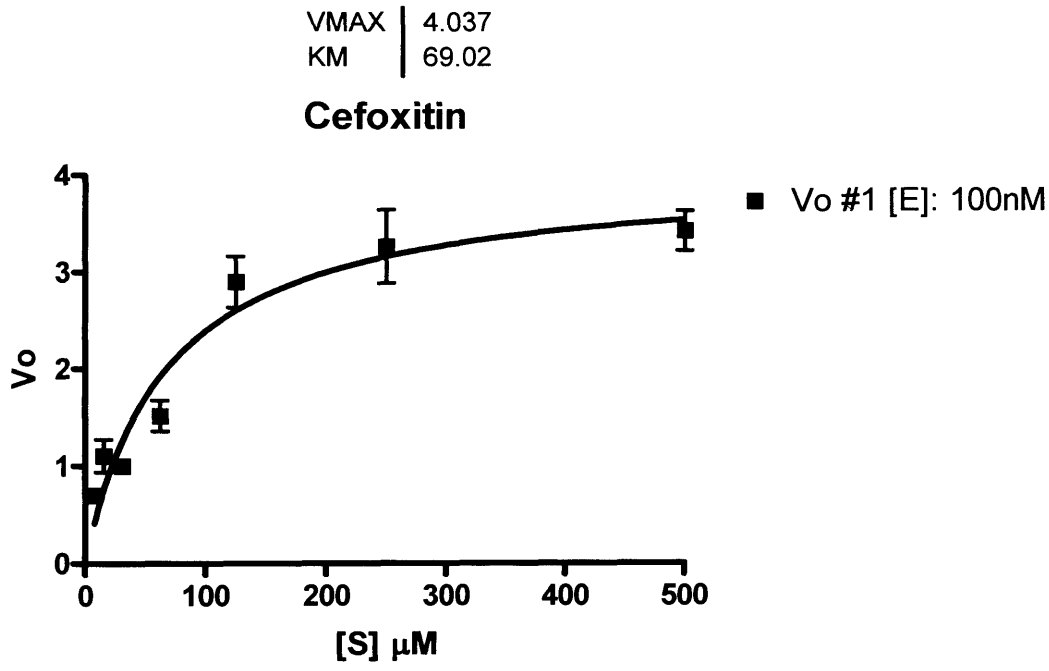

Figure D.7 Hydrolysis of antibiotic cefuroxime by TMB-1

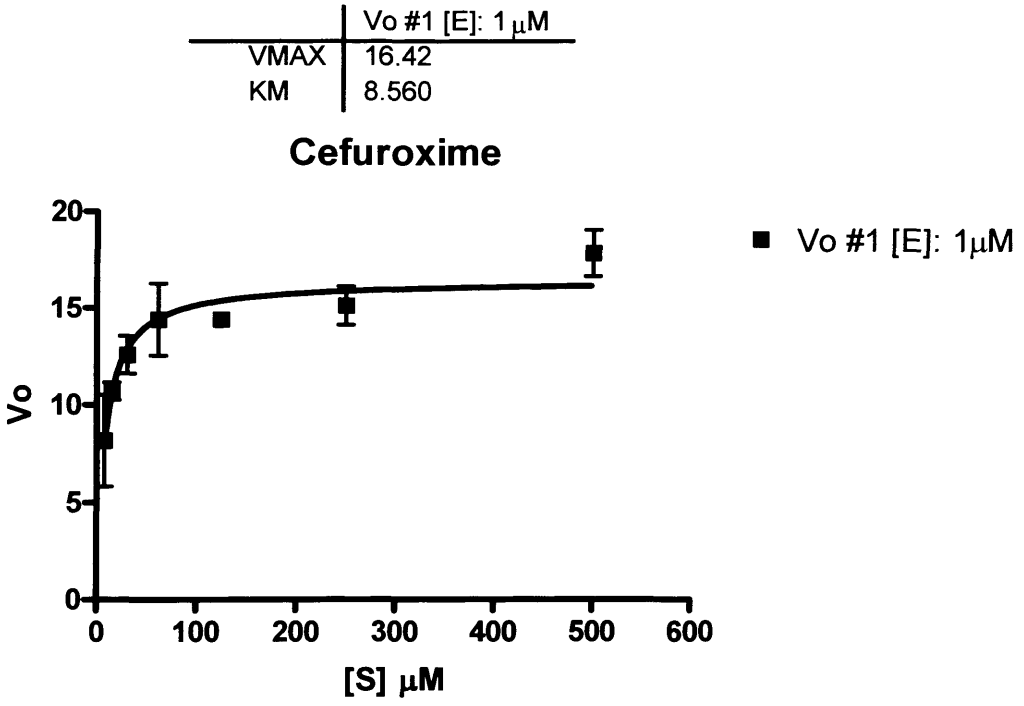

Figure D.8 Hydrolysis of antibiotic piperacillin by TMB-1

	Vo #1 [E]: 100nM
VMAX	6.831
KM	72.13

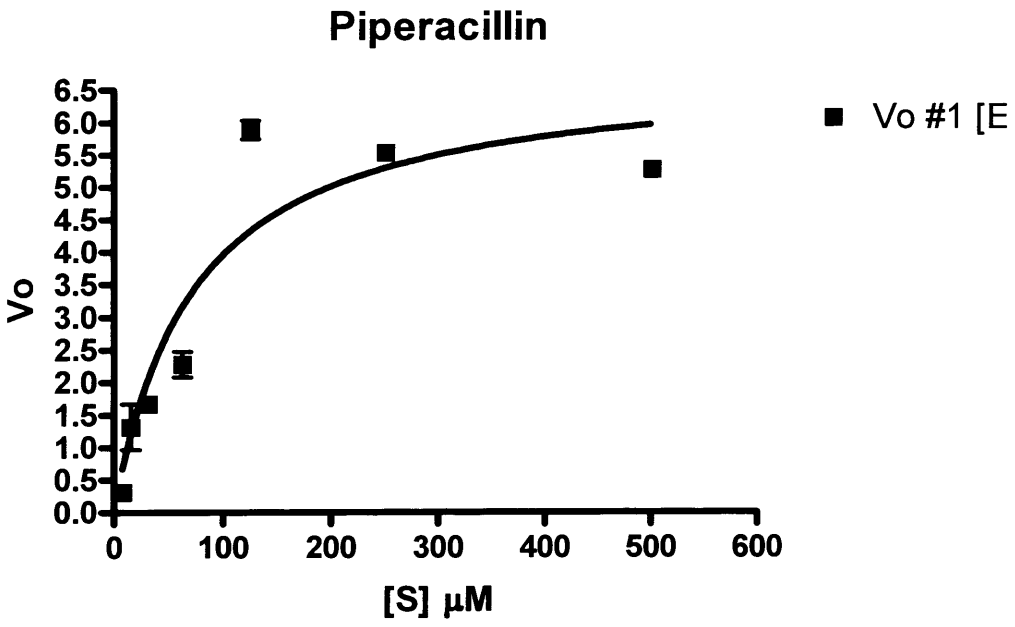

Figure D.9 full class 1 integron (3kb) *bla*$_{TMB-1}$, aac6II and *bla*$_{OXA-4}$ from *Achromobacter xylosoxidans* AES301

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Chapter Nine
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