بسم الله الرحمن الرحيم

Republic of Sudan
Ministry of Higher Education and scientific Research
University of Shendi
Faculty of Graduate Studies and Scientific Research

Phenotypic and Genotypic Characterization of Extended-Spectrum β-lactamases Producing Enterobacteriaceae *in Al-Madinah Al-Monawwara Region, KSA*

A thesis submitted in the fulfilment of the requirement the degree of Doctor of Philosophy (PhD) in Medical Laboratory Science (Microbiology)

By

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July 2018

بسم الله الرحمن الرحيم

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صدق الله العظيم

Dedication

This work is dedicated to

My parent's soul

Brother,

Sisters,

Wife,

My lovely kids,

Friends and

Teachers

To all who has ever taught me anything

Acknowledgements

All thanks to Allah for giving me courage and the determination, as well as guidance in conducting this research, despite all difficulties. Special thanks to my great father for his moral and financial support.Many people have contributed to my success in completing this study. I was fortunate to have an outstanding supervisor, Dr. Mogahid Mohammed El Hassan and Co-supervisor Dr. Amani Osman Shakak for their continuous support of my PhD research project, for their patience, motivation, enthusiasm, and immense knowledge. Their guidance helped me in all the time of research and writing of this thesis. Special thanks to my colleague Dr.Leila M Ahmed for her assistance, encouragement and support during this research, I also must acknowledge the staff of Microbiology Laboratory, Military Hospital, Almadena Almonawarah and the staff of research laboratory, College of Applied Medical Sciences,Taibah University for their help by opinions and advices outstanding experience in this regard. Finally, thanks to my family, my wife who gave me such attention and time to achieve this project.

ABSTRACT

Background:

Reports on extended-spectrum β-lactamases(ESBLs) producers and the genes responsible for ESBL phenomenon are few in Saudi Arabia.

Objectives:

This study aimed to determine the prevalence of ESBL in Al-Mad enah Al-Monawwarah region and characterize the predominant ESBL genes among target, taking into consideration the emergence of (CTX-M) gene in the community.

Methods:

Three hundred and fifty nine (n = 359) gram negative isolates were collected from Prince Sultan Military Hospitals and King Fahad Hospital in Al-Madenah Al-Monawwarah, KSA.Identification of the Enterobacteriacea isolates was done by using conventional biochemical methods and BD phoenix 100 automated system. All the 359 Enterobacteriaceae isolates that identified by BD Phoenix 100 and showed ESBL positive were subjected to the screening disc diffusion test, and followed by confirmatory disc diffusion testing by using Combination Disk and Modified Double Disk Synergy Test according to CLSI guidelines. ESBLs positive strains were tested for the presence of ESBL encoding genes using multiplex PCR with specific primers for the detection of CTX-M,SHV, and TEM genes .

Results:

Out of the total 359 enterobacterial isolates,*Escherichia coli* was identified from 189 (52.6%) and *Klibsiella pneumoniae* from 87 (24.2%). ESBL was demonstrated in 85 (23.7%) of the total 359 isolates.ESBLs were identified only among *E. coli* (66/85; 77.6%), and *K. pneumoniae* (19/85; 22.4%). CTX-M was found to be the most dominant gene (74.1%) followed by TEM (31.8%) and SHV (14.1%). Urine samples

were the most frequent in this study (61.7%) followed by blood (15.1%), pus (8.2%), wound (4.5%), sputum (4.1%), eye swap (2.7%) and high vaginal swap (2.7%).Our results also showed that,the females were more than males and the frequency of age more than 60 years(37.6%) was higher than others also the patients under antibiotics treatment (83.5%) were more than those not under treatment (16.5%) and the ESBL-producing strains were most commonly isolated from ICU 26 (30.6 %).High resistance of ESBL producers was observed among antibiotics belonging to different families including Aztereonam (95.3%), Cephalothin (95.3%), Ampicillin (95.3%), Ciprofoxacin (72.9%), Trimethoprime-Sulfamethaxazole (71.8%), Norfloxacin (68.2%), Levofloxacin (60.0%), Amikacin (33.9%) and Gentamicin (24.7%).

Conclusion:

The study concluded that all ESBL genes were carried by *K. pneumoniae* and *E.*coli with the proved prevalence of CTX-M in Al-Madenah community.This reflects the continuous strategies followed by Enteriobactertiaceaeto evade antimicrobial action.This represent a challenge to the clinicians in this important part of Saudi Arabia as ESBLs, being a cause of outbreaks, is a public health concern.

مستخلص الدراسة

خلفية الدراسة :

التبليغ عن المسببات من البكتريا التي تنتج إنزيم ESBL والجينات المسئولة عن ظاهرة انزيم ESBL قليلة في المملكة العربية السعودية.

اهداف الدراسة : هذه الدراسة تهدف لتحديد إنتشارانزيم ESBL فى منطقة المدينة المنورة وتمييزالجينات السائدة في هذه الدراسة مع الوضع في الاعتبارظهورجين CTX-M في المجتمع

طرق الدراسة: تم عزل 359 من بكتريا سالبة الجرام من نوع الانتروباكتريا من مستشفى الأمير سلطان للقوات المسلحة ومستشفى الملك فهد بالمدينة المنورة في المملكة العربية السعودية وتم التعرف عليها بإستخدام الاختبارات الكيمائية التقليدية والنظام االآلى باستخدام جهاز BD Phoeix 100. جميع إنتيروباكتريا التي تم التعرف عليها بواسطة النظام الآلى والتي أظهرت إجابية لإنزيم ESBL خضعت لإختبارإنتشار القرص الفرزى ثم أتبعتها إختبارات إنتشار القرص التأكيدية طبقا لتوجيهات معهد المختبرات الإكلينكية(CLSI) كما تم فحص البكتريا الاجابية لهذا الانزيم بواسطة اختبار البوليميراز المتسلسل للتعرف على أنواع الجينات CTX-M ، SHV، و TEM.

نتائج الدراسة: وجد من مجموع 359 من بكترياالأنتيروباكتريا بكتريا اشرشيا كولاى وكلبسيلا نمونيا هم الأكثر انتشارا . 189 من نوع اشرشيا كولاى وتمثل 52.6% و 87 من نوع كلبسيلا ويمثل 24.2% ومن مجموع (359) 85 (23.7%) ايجابى لإنزيم ESBL و274 (76.3%) سالبة لإنزيم ESBL وأن من مجموع 85 الإيجابى لإنزيمESBL ، 66 (77.6%) من نوع إشرشيا كولاى و19 (22.4%) من نوع كلبسيلا نيمونيا وتعتبرعينة البول من أكثرالعينات شيوعا 221 (61.17%).كما أن من المشاركين في الدراسة من النساء كانو أكثر من الرجال و الأعمار من 60 سنة وأكثرهم أكثر عرضة للاصابة بالبكتريا التي تحمل انزيم ESBL 32(37.6%) وكذلك المرضى الذين كانوا يستخدمون المضادات الحيوية 71(83.5%) من مجموع المصابين(85). كذلك أكثر المصابين كانو من مرضى العناية المركزة 26(30.6). أكدت النتائج أن الطفرة الجينية CTX-M هى أكثر جين سائد بنسبة 74.1% ثم يتبعها TEM (31.8%) و SHV(14.1%) كما وجد أن البكتريا التي تحمل إنزيم ESBL صارت تقاوم أنواع كثيرة ومختلفة من المضادات الحيوية والتي تشمل ازتريونام ، سيفالوسين، امبسلين بنسبة 95.3% على التوالي، سبروفلوكساسين 72.9% ، ترايمزويريم- سلفاميساكسازول 71.8% ،نورفلوكساسين 68.2% لوفلوكساسين 60% ، أميكاسين33.9% وجنتاميسين 24.7%.

الخلاصة: خلصت الدراسة إلى أن كل جينات إنزيم ESBL كانت محمولة فى بكتريا إشرشيا

كولاى وكليبسيلا نيمونيا. كما برهنت الدراسة على إنتشارالطفرة الجينية CTX-M فى وسط مجتمع المدينة المنورة ويمثل هذا تحدي للأطباء والأوساط الطبية الأخرى مما عكس على تبنى سياسات واستراتيجيات مستمرة لمتابعة هذه المشكلة .

PREFACE

The practical part of this project was conducted at the Research Laboratory, College of Applied Medical Sciences, Taibah University, KSA. Financial supported was received from the Deanship of Scientific Research, Taibah University, Al-Madenah Al-Monawwarah, KSA (Grant number 435/6092). Part of the project's findings were published in a peer-reviewed, ISI Journal with IF (0.54).

1. Mogahid M Elhassan, Hani A Ozbazk, Hassan A Hemeg, **AbdAlla A Ahmed (2**016). Dissemination of CTX-M extended-spectrum β-lactamases (ESBLs) among *Escherichia coli* and *Klebsiella pneumoniae* in Al-Madenah Al-Monawwarah region, Saudi Arabia. International Journal of Clinical and Experimental Medicine. 9 (6): 11051-11057

2. **AbdAlla A Ahmed**, Amani O Shakak and Mogahid M Elhassan (2o18). Detection and reporting of organisms producing extended-spectrum beta-lactamases: survey of laboratories from different Hospitals in Almadenah Almonawarah Region, KSA. (In preperation).

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LIST OF ABBREVIATIONS

Abbreviation	Correspondence
ESBL	Extended Spectrum Beta Lactamase
CTX-M	CefoTaXime
TEM	Temoniera
SHV	Sulf hydril variable
OXA	Oxacillin
PER	Pseudomonas Extended Resistance
VEB	Vietnam Extended-spectrum ß-lactamase
GES	Guyana Extended-Spectrum ß-lactamases
IBC	Institutional Biosafety Committee
VIM	Verona Integron-encoded Metallo- β –lactamase
IRT	Inhibitor-Resistant β-lactamases
MRSA	Methicillin Resistant Staphylococcus Aureus
E.coli	*Escherichiacoli*
Etal	*et alia* (and others)
MDR	Multi Drug Resistance
WHO	World Health Orgnization
OMP	Outer Membrane Permeability
DNA	Deoxyribonucleic Acid
HGT	Horizontal Gene Transfer
UTI	Urinary tract infection
PBPs	Penicillin Binding Proteins
CLSI	Clinical Laboratory Standard Institute
MBL	Metallo-Beta Lactamases
KPC	*K. pneumoniae* Carbapenemase
DD test	Double-Disk test
AIDS	Aquired Immune Deficiency Syndrom
MIC	Minimum Inhibitory Concentration
IS*Ecp1*	Insertion Sequence Elements
IS*CR1*	Insertion Sequence
Tn	Transposons
GNR	Gram Negative Rod
API 20E	Analytical Profile Index
DDST	Double Disc Synergy Test
MHA	Mueller-Hinton agar
AMC	Amoxycillin with clavulinic acid
CA	Ceftazidim
CRO	Ceftriaxone
CTX	Cefotaxime
ATM	Aztreonam
CA	clavulinic acid
S	Sensitive
R	Resistance
I	Intermediate resistance
ATCC	American Type Culture Collection
FDA	Food and Drug Administration

BD	Becton DickinsonPhoenix
PCR	Polymerase Chain Reaction
RFLPs	Restriction Fragment Length Polymorphism
RP	Ceftazidim
AUF	Ceftriaxone
Inc	Incompatibility
CLED	Cystine Lactose Elecrolyte Deficient
XLD	Xylose Lysine Deoxycholate
TSI	Triple Sugar Iron Agar

KOH	Potassium hydroxide
CO2	Carbon dioxide
ID	Identification
AST	Anti Susceptibility test
MDDST	Modified Double Disk Synergy Test
CT	Cefotaxime
CTL	Cefotaxime with clavulanic acid
TZ	Ceftazidime
TZL	Ceftazidime with clavulanic acid
PM	Cefepime
PML	Cefepime with clavulanic acid
CAZ	Ceftazidime
FEB	Cefepime
CTR	Ceftriaxone
CPD	Cefpodoxime
CPM	Cefipime
CRKP	Carbapenemase in *KliebsiellaPnemoniae*
Kbp	Kilobase pair
Tn	Transposons

CHAPTER ONE
INTRODUCTION AND OBJECTIVES

1.1 Introduction

Bacterial infection is the most common cause for hospital visits. In almost all cases of bacterial infections, there is a need to start treatment before the final microbiological results are available. Extended-spectrum beta-lactamase (ESBL) production is an increasing antibiotic resistance problem. Moreover, the production of ESBL byEnterobacteriaceae is an increasing challenge in the treatment of infections. The ESBL type most frequently detected in the last few years is CTX-M, which also has the particular ability to spread in the community (Carlo et al., 2009).Recently, a significant increase in the incidents of ESBL-related infections has been observed throughout the globe (Rupinder *etal.*, 2013) . In Saudi hospitals, the rate of resistance to beta-lactamase grew rapidly in most of the admitted patients (Kadar et al., 2005). This resistance may be due to ESBL.This increasing number of ESBL-producing bacteria is being reported worldwide. Revising the literature regarding this topic still remains few. This study is expected to highlight the problem of ESBLs in KSA. Prevalence, risk factors and situation of resistance will also be highlighted. Keeping these facts in mind, we designed our study to investigate the prevalence of ESBL-producing Enterobacteriaceae in some hospitals in Al-Madina Al-Monawwara area. Hence, this study will be undertaken to find out the antibiotic susceptibility pattern of the isolated pathogenic Enterobacteriaceae from various clinical specimens. Both conventional,automated microbiology and molecular methods will be used for isolation and characterization of ESBL producers. Results were be analyzed by the statistical methods and the final findings will be publishedin abeer reviewed journal Beta-lactamases are enzymes

produced by some bacteria and are responsible for their resistance to beta-lactam antibiotics like penicillins, cephalosporins (are relatively resistant to beta-lactamase), cephamycins, and carbapenems (ertapenem). These antibiotics have a common element in their molecular structure: a four-atom ring known as a beta-lactam. The lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties.Beta-lactam antibiotics are typically used to treat a broad spectrum of gram-positive and gram-negative bacteria. Beta-lactamases produced by gram-negative organisms are usually secreted. The beta-lactamase may be clinically beneficial when orally administered to preserve the natural intestinal flora during the parenteral administration of antimicrobials (Brin et al., 2005, Mirelis B etal, 2003 , DiPersio et al., 1997-2003; Gray et al., 2004).ESBLs are the derivatives of common beta-lactamases (TEM and SHV beta-lactamases) that have undergone one or more amino acid substitutions near the active site of the enzyme, thus increasing their affinity and the hydrolytic activity against third generation cephalosporins and monobactams. Extensive use of newer generation cephalosporins has been the strong factor for the evolution of newer beta-lactamases such as ESBLs. ESBLs are encoded by transferable conjugative plasmids, which often code resistance determinants to other antimicrobial agents such as aminoglycosides. These conjugative plasmids are responsible for the dissemination of resistance to other members of gram negative bacteria in hospitals and in the community (Carlo et al., 2009; EPI-NYT, 2004; Fang et al., 2003; Fey et al., 2003).The development of antimicrobials resistance can be viewed as a global problem in microbial genetic ecology. It is a very complex problem to study, let alone solve, due to the geographic scale, the variety of environmental factors, and the vast number and diversity of microbial participants. ESBLs continue to be a major problem in clinical setups worldwide, conferring resistance against

extended spectrum cephalosporins. Increasing resistance to third and fourth generation cephalosporins has become a cause of concern (Smith et al., 2005; Shailaja et al., 2004; Tambekar et al., 2006; Das et al., 2006). Enterobacteriaceae that produceESBLs have emerged as significant pathogens. First reported in the mid-1980s, they were mainly found in *Klebsiella pneumoniae* and *E. coli* although they can now be found in many other species.Enterobacteriaceae strains that harbor ESBLs were frequently associated with resistance to aminoglycosides and ciprofloxacin (Paterson et al., 2005; Soge et al., 2005).

1.2 Hypothesis of the Study

CTX-M β-lactamases are considered a paradigm in the evolution of drug resistance mechanism. Incorporation of different chromosomal *bla*CTX-M related genes (*bla*SHVand *bla* TEM) from different species of *Enterobacteriacae*. Hence, the situation in a region like Almadenah Almonawarah may reflect with other findings.

1.3 Rationale

- ESBL-producing organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists.
- *In Saudi hospitals, the rate of resistance to beta-lactamase grew* rapidly in the most of the admitted patients.
- This resistance may be due to ESBL. Few dataon type of circulating ESBL is available at national level.
- This increasing number of ESBL-producing bacteria is being reported world wide
- This study is expected to highlight the problem of ESBLs in KSA.
- Prevalence, risk factors and situation of resistance will also be highlighted.
- Keeping these facts in mind, we designed our study to investigate the prevalence of ESBL-producingEnterobacteriaceae in some hospitals in Al-Madinah Al-Monawarah area.
- Hence, this study will be undertaken to find out the antibiotics susceptibility pattern of the isolated pathogenic Enterobacteriaceae from various clinical specimens.
- Both conventional,automated microbiology system and molecular methods will be used for isolation and characterization of ESBL producers.

- Results will be analyzed by the proper statistical methods and the final findings will be published in a beer-reviewed journal.

1.4 Research Questions

- What are the most common pathogens producing ESBL in Al-Madinah Al-Munawwarah?
- What is the real situation of ESBLs in Al-Madīnah Al-Munawwarah?
- What is the dominant gene(s) that is (are) responsible for ESBLs?

1.5 Objectives of the Study

1.5.1 General Objective

The present study aims to detect phenotypic and genotypic characterization of ESBLsproducing-Enterobacteriaceae in Al-Madinah Al-Monawwara Region, KSA.

1.5.2 Specific Objectives

1. To isolate and identify ESBL in different Hospitals in Al Madinah Al Monawarah using conventional and automated method.
2. To determine antibiotic profile of ESBL against different antibiotics.
3. To detect different genes responsible for ESBL Phenomenon (*TEM, SHV, CTXM*) in Al Madenah Al Monawarah Region.
4. To evaluate the frequency of different genes responsible for ESBL in Almadenah Al Monawarah.

CHAPTER TWO

LITRATURE REVIEW

2.1 History of Antibiotic Resistant Bacteria

Humans have been plagued with the threat of bacterial infection since before the dawn of recorded history, and have thus always had a keen interest in finding a suitable treatment for these maladies. Folk medicine is replete with accounts of the use of various materials for the treatment of bacterial infection (moldy bread, moldy cheese, plant and animal preparations), which we now assume were effective due to the presence of some unknown antibacterial agent (Toder, 2008). In 1928 Alexander Fleming observed that culture plate on which Staphylococci were being grown had become contaminated with a mold of the genus Penicllium and that bacterial growth in the vicinity of the mold had been inhibited. He isolated the mold in pure culture and demonstrated that it produced an antibacterial substance Penicillin (Abraham and Chain,1940). Abraham and Chain (1940) have shown that certain bacteria produce an enzyme named penicillinase, which destroy penicillin (Woodruff and Foster,1945). Within a few years of introduction of penicillin into clinical use, penicillinase producing *Staphylococcus aureus* started to proliferate in hospitals. To overcome this problem, penicillinase resistant penicillins came into picture. Shortly afterward,the broad spectrum penicillins and first generation cephalosporins were introduced.They remained a first line of defense against microbes for over 20 years, before resistance due to Beta-lactamases produced by gram negative bacilli became a serious problem (Medeiros,1997).To counter this threat, the pharmaceutical industry marketed six novel classes of beta lactam antibiotics (cephamycins, oxyimino cephalosporins, carbapenems, mono bactams and clavam and penicillianic acid sulfone inhibitors) within a relatively

short span of 7-8 years. Although, novel beta-lactamases had emerged gradually after the introduction of new beta lactam agents, their number and variety accelerated at an alarming rate (Chaudhary and Aggarwal,2004).Beta-lactamases were present in bacteria long before the introduction of penicillins (Woodruff and Foster,1945), and genes encoding these ancient enzymes were originally located on the bacterial chromosome (Hanson *et al* .,1999; Yusha *et al*.,2010). Furthermore, these enzymes are inducible and constitutively expressed in low quantities. In 1965, the first report of a plasmid-encoded beta-lactamase in a Gram negative bacterium appeared from Greece (Datta and Kontomichalou,1965). Beta-lactamase producing bacteria are increasing in number and causing more sever infections (Shobha *et al*.,2007; Andrews,2009).Development of antibiotic resistance was first reported in animal models in 1940s and subjectively reported among patients in the 1970s (Rekha *et al*., 2009).Today drug resistant strains of *Mycobacterium tuberculosis* are threatening to outbreak in one of the world's most prevalent infectious diseases (Bari *et al*., 2008).

2.1.1 Antibiotic Resistance

Antibiotic resistance is of utmost importance for the clinical impact of ESBL-producing bacteria. Ameta-analysis showed increased mortality and delay in effective antibiotic use in ESBL-related bacteremia (Schwaber *et al*.,2007). Indicating the importance of constant surveillance for an antibiotic resistance pattern in organisms with ESBLs. ESBL-producing bacteria are resistant to almost all beta-lactam antibiotics, except carbapenems, as indicated by their definition.

In addition, most ESBL-producing bacteria, particularly those with the TEM, SHV, and CTX-M genotypes, exhibit co resistance to aminoglycosides, tetracyclines, and sulfonamides(Canton *et al*.,2006).

2.1.2 Mechanisms of Resistance

Multi-drug resistance Enterobacteriaceae is associated with increased mortality compared with their susceptible counterparts, which is mainly related to the intrinsic difficulties of therapy of MDR isolates (Rottier *et al*., 2012; Vardakas, 2013).

2.1.2.1 Efflux of Antibiotics from Bacteria

Efflux pumps play a major role in antibiotic resistance and also serve other functions in bacteria such as the uptake of essential nutrients and ions, excretion of metabolic end products and deleterious substances as well as the communication between cells and environment (Li and Nikaido,2004).

2.1.2.2 Outer Membrane Permeability

The OM of Gram negative bacteria is a barrier to both hydrophobic and hydrophilic compounds. By combining a highly hydrophobic lipid bilayer with pore forming proteins of specific size-exclusion properties, the OM acts as a selective barrier. The permeability properties of this barrier have a major impact on the susceptibility of the microorganism to antibiotics, which are essentially targeted at intracellularprocesses.Small hydrophilic antibiotics, such as, β-lactams, use the pore forming proteins (water filled channel proteins embedded in the outer membrane, e.g., OmpF in *E. coli* and OprD in *Pseudomonas aeroginosa*) to gain access to the cell interior, while macrolides and other hydrophobic antibiotics diffuse across the lipid bilayer. The existance of antibiotic-resistant strains in a large number of bacterial species due to modifications in the lipid or protein composition of the OM indeed highlights the importance of the OM barrier in antibiotic sensitivity (den Engelsen *et al;* 2009).

2.1.2. 3 Target Modifications

This mechanism is based on alterations of bacterial sites that are targeted by antibiotics and thus preventing the antibiotic from binding to its site of

action. For example fluoroquinolone resistance is attributed to mutations within the drug's target (DNA gyrase and topoisomerase) (Livermore,2003).

2.1.2.4 Enzymatic Modification of the Antibiotic

Enzymes that modify antibacterial antibiotics are divided into two general classes: β-lactamase that degrade antibiotics and Others (including the macrolide and aminoglycoside-modifying proteins) that perform chemical transformations to render the antibiotic inefficient (Livermore,2003).

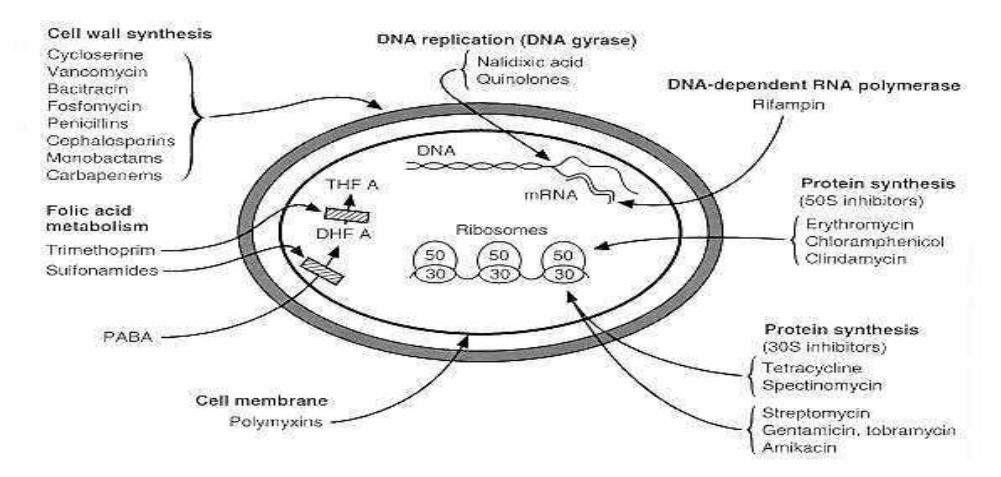

Figure1. Illustration of different mechanisms of antibiotics resistance.

2.1.2.5 Inherent Resistance

Bacteria may be inherently (naturally) resistant to an antibiotic. For example, an organism lacks a transport system for an antibiotic; or an organism lacks the target of the antibiotic molecule; or, as in the case of Gram-negative bacteria, the cell wall is covered with an outer membrane that establishes a permeability barrier against the antibiotic(Livermore, 2003).

2.1.2.6 Acquired Resistance

Several mechanisms are developed by bacteria in order to acquire resistance to antibiotics. All required either the modification of existing genetic material or the acquisition of new genetic material from another source(Livermore, 2003).

2.1.2.7 Vertical Gene Transfer

Once the resistance genes have developed, they are transferred directly to all the bacteria's progeny during DNA replication. This is known as vertical gene transfer or vertical evolution.

2.1.2.8 Horizontal Gene Transfer

Lateral or horizontal gene transfer (HGT) is a process where genetic material contained in small packets of DNA can be transferred between individual bacteria of the same species or even between different species.There are at least three possible mechanisms of HGT, equivalent to the three processes of genetic exchange in bacteria. These are transduction, transformation and conjugation(Toder, 2008).

2.1.2.8.1 Conjugation

Occurs when there is direct cell-cell contact between two bacteria (which need not be closely related) and transfer of small pieces of DNA called plasmids takes place. This is thought to be the main mechanism of HGT(Toder, 2008).

2.1.2.8.2 Transduction

Transduction occurs when bacteria-specific viruses (bacteriophages) transfer DNA between two closely related bacteria (Toder, 2008).

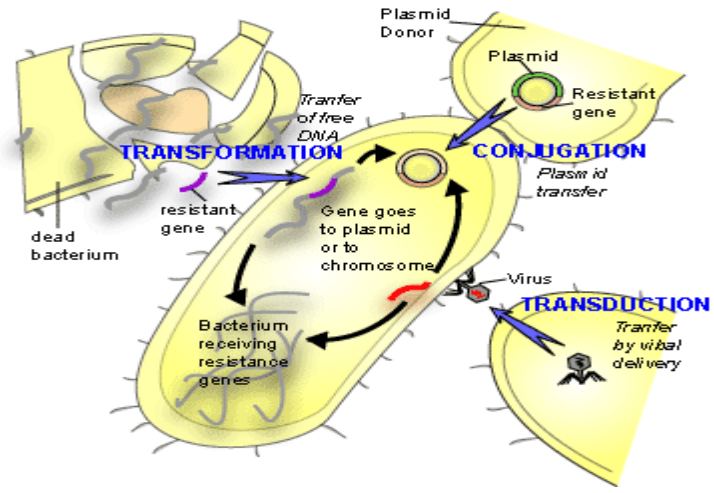

Figure2. Mechanism of drug resistance by horizontal transfer(Toder, 2008).

2.1.2.8.3 Transformations

It is a process where parts of DNA are taken up by the bacteria from the external -environment. This DNA is normally present in the external environment due to the death and lysis of another bacterium(Toder, 2008).

2.2 Beta Lactam Antibiotics

β-lactam antibiotics are a broad class of antibiotics that include penicillin derivatives, cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors, that is, any antibiotic agent that contains a β-lactam nucleus in its molecular structure. They are the most widely-used group of antibiotics available (Llarrull *et al*.,2010). Beta lactam antimicrobials are the most common treatment for gram positive,gram negative and anaerobic bacterial infection (Ambler,1980; Kotra *et al*; 2002; Holten and Onusko, 2000).

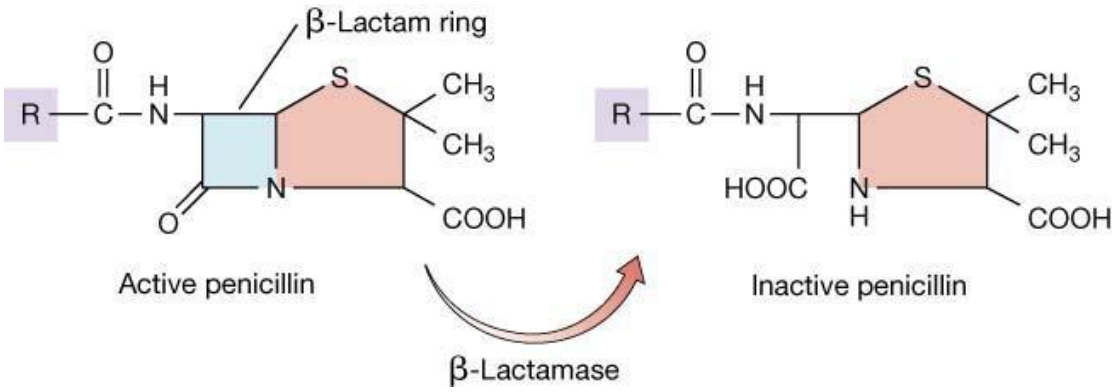

Figure 3. Penicillin Showing beta lactam ring destruction by beta lactamases enzyme

2.2.1 Clinical use of Beta Lactam Antibiotics

β-lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms.At first,β-lactam antibiotics were mainly active only against Gram-positive bacteria,yet the recent development of broad-spectrum β-lactam antibiotics active against

various Gram-negative organisms has increased their usefulness (Llarrull*et al*,2010).

2.2.2 Mode of Actions

β-Lactam antibiotics are bactericidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms.The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs) (Llarrull *et al*.,2010).

2.2.3 Modes of Resistance

By definition, all β-lactam antibiotics have a β-lactam ring in their structure. The effectiveness of these antibiotics relies on their ability to reach the PBP intact and their ability to bind to the PBP. Hence, there are 2 main modes of bacterial resistance to β-lactams, as discussed below (Qic *et al*.,2010).The first mode of β-lactam resistance is due to enzymatic hydrolysis of the β-lactam ring. If the bacteria produces the enzymesβ-lactamase or penicillinase,these enzymes will break open the β-lactam ring of the antibiotic, rendering the antibiotic ineffective. The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired via plasmid transfer, and β-lactamase geneexpression may be induced by exposure to beta-lactams (Kalp *et al*.,2009; Fenosa *et al*.,2009).The second mode of β-lactam resistance is due to possession of altered penicillin-binding proteins. β-lactams cannot bind as effectively to these altered PBPs, and, as a result, the β-lactams are less effective at disrupting cell wall synthesis. Notable examples of this mode of resistance include Methicillin-Resistant *Staphylococcus aureus*(MRSA) and Penicillin-Resistant *Streptococcus pneumoniae* (Kalp *et al*.,2009; Fenosa *et al*.,2009).

2.3 Extended Spectrum Beta Lactamases

ESBLswere initially identified in the year 1983 by Knothe *et al* (Bradford,2001). Beta-lactamases (>500) > 50% ESBLs.ESBLsare rapidly evolving group of beta-lactamase enzymes, with the ability to hydrolyse and cause resistance to the oxyiminocephalosporins (i.e. cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cefepime) and monobactams (i.e. aztreonam), but not the cephamycins (i.e. cefoxitin and cefotetan) or carbapenems (i.e. imipenem, meropenem, and ertapenem), produced by the Gram negative bacteria more commonly in *E.coli* and *K.pneumoniae* (Lal *et al.*,2007; Rupp and Fey,2003; Peirano and Pitout,2010). Beta-lactamases are among the most heterogeneous group of resistance enzymes.These globular proteins are composed of alpha-helices andβ-pleated sheets.Despite a significant amount of amino acid sequence variability, beta lactamases share a common overall topology (Perez *et al*.,2007).

2.3.1 Mechanisms of Action

A beta-lactam ring of penicillin-like drugs (Chaudhary and Aggarwal,2004; Paterson,1999).A point mutation which alters the configuration around the active site of TEM and SHV type enzymes that specify resistance to ampicillin and had a new capability to hydrolyze a broader spectrum of beta lactamdrugs(Philippon,1989). Medical use of antibiotics can considerably accelerate the selection pressure for diversification and dissemination of mutant extended spectrum beta lactamase (Farkosh, 2007).ESBLs have serine at their active site and attack the amide bond in the lactam ring of antibiotics causing their hydrolysis (Chaudhary and Aggarwal, 2004).common mechanism of bacterial resistance to beta-lactam antibiotics is the production of beta-lactamase enzymes that break down the structural

2.3.2 Synthesis and Mode of Transfer

The synthesis of β- lactamases is either chromosomal (constitutive), as in *P.aeruginosa*, or plasmid mediated (inducible), as in *E.coli* and *S.aureus* (Livermore, 1995; Rayamajhi *et al*., 2008; Rupp and Paul, 2003). Plasmids are a major cause of bacterial resistance spreading (Peirano and Pitout, 2010). The genes encoding these β lactamases are often located on large plasmid (80kbp) that also encode genes for resistance to other antibiotics, including aminoglycosides, tetracycline, sulfonamides, trimethoprime and chloramphenicol which can easily be transferred between isolates (Sasirekha *et al*.,2010 ; Perez, 2007).

2.3.3 Global Epidemiology

Epidemiology of ESBLs genes are rapidly changing and shows marked geographic differences in distribution of genotypes of CTX-M- β-lactamases (Coque et al,2008).Rupp ME in 2003 encoded- in many parts of the world (10-40%) of strains of *E.coli* infrequentESBLs, PeculiarESBLs and *K.pneumoniae* express ESBLs (Rupp and Paul,2003) also noted in the introduction, ESBLs were first described in 1983 from the Germany. The success of the CTX-Ms over the classical ESBL-enzymes SHVs and TEMs is linked to the way by which CTX-M enzymes are spread. Through mobile genetic elements, resistance genes disseminate within the same species and also between bacteria of different species (Coque et al,2008).The prevalence of ESBLs in Europe is higher than in the USA but lower than in Asia and South America (Girlich *et al*.,2004).

2.3.3.1 European Countries

ESBL-producing organisms were first detected in Europe. Although the initial reports were from Germany (Knothe*et al*., 1983**)** and England (Du Bois *et al*., 1995**).**

first large outbreak in France to be reported occurred in 1986 (Brun - Buisson etal,1987).In northern France, the proportion of *Klebsiella pneumoniae* isolates which were ESBL-producing fell from 19.7% in 1996 to 7.9% in 2000 (Albertini,2000**).**It is noteworthy however that 30.2% of *Enterobacter aerogenes* isolates in 2000 were ESBL producers (Albertini *et al*.,2000).Outbreaks of infection with ESBL-producing organisms have now been reported from virtually every European country.There is considerable geographical difference in the occurrence of ESBLs in European countries. Within countries, hospital-to-hospital variability in occurrence may also be marked ((Babini *et al*.,and Livermore, 2000).

In a 1997–1998 survey of 433 isolates from 24 intensive care units in western and southern Europe, 25% of klebsiellae was found to possessed ESBLs (Babini and Livermore,2000).

A similar survey was performed by the same group in 1994; the overall proportion of klebsiellae which possessed ESBLs did not differ significantly between the two time periods, but the percentage of intensive care units which recorded ESBL-producing klebsiellae rose significantly from 74% to _90% (Babini *et al*.,and Livermore,2000).

Another large study from more than 100 European intensive care units found that the prevalence of ESBLs in klebsiellae ranged from as low as 3% in Sweden to as high as 34% in Portugal (Hanberger *et al*., 1999).

A third study, which included both intensive care unit and non-intensive care unit isolates from 25 European hospitals, found that 21% of *Klebsiella pneumonia* isolates had reduced susceptibility to ceftazidime (usually indicative of ESBL production, although it is acknowledged that other mechanisms of resistance may be responsible (Fluit*et al*.,2000).

However, outbreaks with SHV-type ESBLs have also been described (Jacoby *et al.,* 1997).

In Turkey, a survey of *Klebsiella* spp. from intensive care units from eight hospitals showed that 58% of 193 isolates harbored ESBLs (Gunseren *et al*., 1999).

A study performed in Turkey showed a prevalence of 21% ESBL producers among *E.coli* causing community acquired urinary tract infection (UTI) during 2004 and 2005 (Coque *et al*.,2008).In Norway, a prospective survey of clinical *E. coli* isolates with reduced susceptibility to oxyimino-cephalosporins demonstrated the dominance of CTX-M-15 (46%) and CTX-M-9-like (30%) enzymes among ESBL-positive *E. coli* and of SHV-5 (47.4%) and SHV-2 (21.0%) among ESBL-positive *K. pneumonia* isolates (Coque *et al*.,2008). In Italy, the prevalence of ESBL producers among clinical isolates has also increased over the past ten years.The most prevalent ESBLpositive species are *E. coli* among hospitalised patients and *P.mirabilis* among outpatients. A predominance of TEM enzymes (45.4%), SHV-12, and the emergence of non-TEM, non-SHV enzymes (CTX-M-type in *E. coli* and *K. pneumoniae*, and PER-type in *P. mirabilis*) has been described. More recent studies performed in single institutions showed the frequent recovery of CTX-M-15-producing *E. coli* and other variants from this group such as CTX-M-1 and CTX-M-32 (Caccamo *et al*.,2006).The prevalence of ESBLs is over 10% in Hungary, Poland,Romania, Russiaand Turkey. *K. pneumoniae* is the most frequent ESBL-producing species in Hungary and Russia,and an increase in the percentage of ESBL producers among *K. pneumoniae*isolates has been reported from Poland, Turkey, Bulgaria, and Romania (Edelstein *et al*.,2003). The emergence and wide spread of the CTX-M-15 enzyme in most European countries, including those with previous low rates of ESBLs, is one of the most relevant findings associated with the current epidemiology of ESBL in Europe (Lytsy *et al*., 2008; Oteo *et al*., 2006). Percentage of organisms expressing ESBL

phenotype in the Meropenem Yearly Susceptibility Test Information Collection study in 1997-2003, the highest rate among *K. pneumoniae* isolated is seen in Eastern Europe and South America, where more than 50% of isolates are potential ESBL producers this contrasts with North America and Northern Europe, where only 12.3% and 16.7% of the isolates, respectively, are potential ESBL producer (Turner *et al*., 2005).

2.3.3.2 AsianCountries

Asia probably has a long history of the occurrence ofESBL-producing bacteria (Kim *et al*.,2007). There were, a number of sporadic reports of ESBLs, notably of the SHV-2 type, from China in 1988 (Rupp *et al*.,2003).ESBL phenotypes were reported for three centres in northern Taiwan, contributing to the 1998.2002 SENTRY programme, with overall rates of ESBL production of 13.5% in *K. pneumoniae* and 5.6% in *E. coli* (Turner *et al*., 2005).ESBLs mediated resistance in *Klebsiella spp*ranged from 20-40% throughout Southeast Asia, China and Japan (Rupp *et al*., 2003). CTX-M-15 has probably been present in India for some considerable time, and is present in both *E. coli* and *Klebsiella spp.* at a high frequency and it is assumed that spreads of CTX-M-15 from India to other countries is more likely. Two very early members of the CTX-M group were identified in Japan: TOHO-1 and -2. These have not spread or evolved *de novo* outside Japan, except for one reported single isolate producing TOHO-1 from Argentina (Bonnet, 2004). The pattern of ESBL genotypes in Japan is quite different from that seen in surrounding countries, although universally successful types, e.g., CTX-M-14, have recently become more common *(*Hirakata *et al*.,2005) Both India and Pakistan have reported high rates of ESBLs since the 1990s (Grover *et al*.,2006;Mathai *et al*., 2002).With the populations of India and China these two countries surely represent the largest reservoirs of CTX-M ESBL genes in the world. Increasing travel and trade will contribute to

the worldwide spread of locally evolved CTX-M genotypes (Hawkey *et al*., 2008). Prevalence of ESBLs vary from country to country, hospital to hospital even very closely related region. There were several studies in India in 2002,2007, 2007, 2008 were 60%, 60.9%, 46.5%, 51.4% in *E.coli* respectively (Mathai *et al*., 2002; Sharma *et al*., 2007; Shivaprokasha *et al*., 2007; Varaiya *et al*., 2008).In India in 2006 CTX-M -15 was 73%. In 2010 there were different studies from different countries e.g. in Iran it was 96%, ESBLs where SHV, CTX-M, TEM, PER was 26%, 24.5%, 18%, 7.5% respectively, in Korea *E.coli* and *Klebsiella* were 17.7% and 26.5% respectively. In India it was 61.1% and 40.6% for *E.coli* and *Klebsiella* respectively (Sasirekha, 2010), another study in India ESBLs for 70% and 60% for *E.coli* and *Klebsiella* respectively, where TEM, SHV were 56% and 60% respectively (Sharma , 2010).

A previous study done in Kuwait at 2005 showed that of the 3,592 bacterial isolates, 264 (7.5%) and 185 (5.2%) were positive for ESBL production by the VITEK 2 and E test, respectively. All the ESBL-producing *P.aeruginosa* identified by VITEK 2 gave indeterminate results by E test. Prevalent ESBL producers, identified by the VITEK 2 versus E test, respectively, were: *Citrobacter spp*. (15 vs. 3.2%), *K. pneumoniae* (12.2 vs. 11.4%), *Enterobacter spp*. (12 vs. 3%), *E. coli* (6.5 vs. 5.6%), *P. aeruginosa* (6.5 vs. 0%) and *Morganella spp.* (2 vs. 1%). The most common infection associated with ESBL-producing pathogens was urinary tract infection (68.2%), followed by wound infection (14.4%) and bloodstream infection (6.1%).Conclusion: The result of this study showed a relatively high prevalence of clinically significant ESBL producers among the Enterobacteriaceae and *Pseudomonas spp*. at our teaching hospital. The VITEK 2 identified a higher prevalence of ESBL strains than the E test (Wafaa *et al*., 2005).

A study done in Saudi Araibia,during the period August 2003 to October 2004, to evaluate the prevalence and antimicrobial susceptibility of ESBLs producing gram-negative organisms isolated from patients with UTI showed that, the ESBL-producing strains were most commonly isolated from patients with indwelling Foley's catheter 131 (64.2%) and those in the long-term care ward 90 (44.2%). Only 26 (12.7%) ESBL-producing isolates were from outpatients. More than 89% of the ESBL producers were susceptible to imipenem and meropenem. Amikacin and piperacillin/tazobactam were active against 68% and 45% of the isolates. Susceptibility to gentamicin and ciprofloxacin was 22.5% and 14% respectively the least active antibiotic was cefepime (11.8%) (Kadar *et al*., 2005).

In Kuwait a study was carried out, to determine the spectrum of microbial etiology and antibiotic resistance pattern of the uropathogens that cause urinary tract infections in 2 large teaching hospitals over a period of 1 year during the period of January to December 2002. It revealed that amikacin provided the widest coverage amongst all the antibiotics tested followed by ciprofloxacin, gentamicin and piperacillin-tazobactam. For the gram-negatives, high resistance (26–63%) to the beta lactam antibiotics was noted, especially to ampicillin, amoxicillin-clavulanic acid, cephalothin and cefuroxime. Resistance to trimethoprim-sulfamethoxazole was also high (Noura *et al*., 2005).Reports from Bouchillon et al of their study from 31 Centers in 14 Countries to study Regional Incidence ofESBL Producing Enterobacteriaceae during the period from Jan 2001 to March 2002, revealed that the highest rates of ESBL occurrence (*K. pneumoniae* plus E. coli) per country were Egypt, 40.9%; Greece, 33.2%; Italy, 18.0%; South Africa, 17.3%; Lebanon, 15.7%; Turkey, 13.2%; Portugal, 9.8%; Spain, 6.8%; Austria, 3.9%; Belgium, 3.8%; Germany, 3.6%; The Netherlands, 2.0%; Switzerland,

1.9%; France, 0.0% (Bouchillon *et al*.,2002). Ibukun Aibinu et al., investigated the occurrence of ESBL enzymes in isolates of Klebsiella spp and *E.coli* from various health institutions in Lagos, Nigeria during the period from December 2000 to October 2001, 356 isolates of Klebsiella sppand *E.coli* (156) were investigated for ESBL production. These isolates were obtained from the Microbiology laboratories of 7 hospitals, Seventy four (20.8%) were found to be ESBL-producers using the double-disk test (DD test). Amongst the ESBL-producers, *K. pneumoniae* was the most represented 60.8%, followed by E.coli 31.1% (Ibukun *et al*.,2003). Another study done in Central African Republic during the period of January 2003 to March 2005 regarding ESBLs Enterobacteriaceae showed that 4% ESBL-producing strains These strains were associated with urinary tract infection urinary tract infection (UTI), pneumonia in an AIDS patient, wound infection, vaginal or intestinal colonization, and ear infection they found that 11 isolates were more resistant to cefotaxime (MIC [greater than or equal to] 256 [micro]g/ml) than to ceftazidime (MIC [less than or equal to] 128 [micro]g/ml), which suggests CTX-M-type enzymes.

2.3.3.3 African Countries

Several outbreaks of infections with ESBL-producing Klebsiellahave been reported from South Africa (Cotton *et al*., 2000 and Karas *et al* .,1996),but no national surveillance figures have been published. However, it has been reported that 36.1% of *Klebsiella pneumoniae* isolates collected in a single South African hospital in 1998 and 1999 were ESBL producers(Bell *et al*.,2002). Outbreaks of Klebsiellainfections with strains resistant to third-generation cephalosporins have been reported in Nigeria and Kenya without documentation of ESBL production (Akindele *et al*.,1997).A novel CTX-M enzyme (CTX-M- 12)

has been found in Kenya (Kariuki etal.,2001. Characterization of ESBLs from South Africa has revealed TEM and SHV types (especially SHV-2 and SHV-5) (Hanson *et al*.,2001).

2.3.3.4 American Countries and Australia

First reports of ESBL-producing organisms in the United States occurred in 1988 (Jacoby *et al*.,1988). CTX-M-type ESBLs have recently been described in the United States and Canada (Boyd *et al*., 2004). In 1988 and 1989 isolates of *Klebsiella pneumoniae* from Chile and Argentina were reported as harboring SHV-2 and SHV-5 (Casellas *et al*., 1989).In 1989 an outbreak of multiresistant *Salmonella enterica* serovar Typhimurium infections occurred in 12 of 14 Argentinian provinces. From these isolates a new non-SHV, non-TEM ESBL named CTX-M-2 was identified (Bauernfeind *et al*.,1996).Other CTX-M enzymes (CTX-M-8, -9, and -16) have been discovered in Brazil (Bonne**t** *et al*., 2000).

Reports of ESBL-producing organisms also exist from Central America and the Caribbean Islands(Cherian *et al*.,1999 ; Diekema *et al*.,1999 ; Gonzalez-Vertiz *et al*., 2001)**.** In the last decade, ESBL-producing organisms have been detected inevery state of Australia and in the Northern Territory(Bell *et al*.,2002 ; Eisen *et al*.,1995 and Howard *et al*., 2002).Outbreaks of infection have occurred in both adult and pediatric patients. Overall, it appears that the proportion of *Klebsiella pneumoniae* isolates which are ESBL producers in Australian hospitals is about 5% (Bell *et al*.,2002).

2.3.4 Genetic Characteristics

Genes harboring ESBLs are associated with several specific genetic structures. A variety of mobile genetic elements,such as transposons, insertion sequences, and integrons, play important roles in the dissemination of ESBL genes.TEM-type ESBL genes are acquired by the

mutation of plasmid-mediated, parent TEM-1 and -2 genes, and the main producer of TEM-type ESBLs is *E.coli*; these genes occur within the earliest bacterial transposons identified (Livermore *et al*.,1995 and Poirel, 2008). SHVtype ESBL genes are the derivatives of chromosomal, parent SHV-1 genes, which occur mainly in *K. pneumoniae (*Babini GS etal.,2008).and are likely acquired by the role of insertion sequences from chromosome to plasmid(Poirel*etal*.,2008).

Notably, TEM- and SHVtype ESBL genes located in the integron structures have never been identified (Poirel *et al*.,2008).The spread of CTX-M-type ESBL genes is associated with more complicated mobile elements, compared to that of TEM and SHV ESBL genes. CTX-M ESBL genes are not derivatives of *K. pneumoniae* or *E. coli* that contain original genes, as compared to TEM or SHV ESBL genes. CTX-M genes originate from the chromosomal lactamase genes of *Kluyvera* species, which are environmental bacteria found worldwide, and are captured mainly by insertion sequence elements translocated from chromosome to plasmid(Canton *et al*.,2006). Original beta-lactamase genes of *Kluyvera* species are identified in most CTX-M subgroups(Canton *et al*.,2006).This differential origin might be involved in the characteristic spread of CTX-M ESBL genes, that is, an "allodemic"pattern of spread.Many CTX-M ESBL genes are associated with integron structures that contain insertion sequences.Well-studied, CTX-M-associated insertion sequence elements include IS*Ecp1* and IS*CR1*, which are involved in the mobilization of CTX-M genes by a recombination mechanism (Novais *et al*.,2006 ; Poirel *et al*.,2005 and Toleman *et al*.,2006). In addition, integron structures bearing insertion sequences and CTX-M genes can be linked to transposon elements, such as from the Tn*21* family, which has been intensively studied. Transposons of the Tn*21* family are disseminated

worldwide in both environmental and clinical bacteria.(Canton *et al*., 2006 and Novais *et al*.,2006) These highly efficient mobile genetic elements may have influenced the rapid and easy dissemination of CTX-M ESBL genes.An antibiotic resistance plasmid itself is responsible for the efficiency of gene transfer, as well as the mobile genetic elements described above. It has been shown that ESBL gene-bearing plasmids can be transferred to different bacterial species by conjugation (Palucha *et al*.,1999 and Baraniak *et al.,* 2009).Previous studies have shown that TEM and SHV ESBL genes are associated with plasmids belonging to a few specific incompatibility (Inc) groups Inc (Canton *et al*.,2006).contrast, CTX-M ESBL genes are carried by plasmids belonging to a variety of Inc groups including narrow- and broad-host-range types(Canton *et al*.,2006 and Carattoli *et al* 2009).CTX-M-15 genes are located mainly on plasmids belonging to the IncF group(Carattoli *et al*.,2009).Interestingly, a recent study has described the diversity of ESBL gene-bearing plasmids, including SHV types (Diestra *et al*., 2009).It was reported that a mosaic plasmid has been identified from a clonal CTX-M-producing *E. coli* isolate, suggesting genetic interactions among different plasmids (Lavollay *et al*., 2006).

2.3.5Classification of β- lactamases

Because of the diversity of enzymatic characteristics of the many β-lactamases discovered so far, many attempts have been made to categories and classify them since the late 1960s. These classifications involve two major approaches: the first and older one is based on the biochemical and functional characteristics of the enzyme;the second approach is based on the molecular structure of the enzyme (Bush, Jacoby *and* Medeiros,1995).

2.3.5.1 Functional Classification

2.3.5.1.1 Group 1

Cephalosporinase, molecular Class C *(not inhibited by clavulanic acid)*Group 1 is cephalosporinases not inhibited by clavulanic acid, belonging to the molecular class C(Bush, Jacoby and Medeiros, 1995).

2.3.5.1.2 Group 2

Group 2 are penicillinases, cephalosporinases, or both inhibited by clavulanic acid,corresponding to the molecular classes A and D reflecting the original TEM and SHV genes.However, because of the increasing number of TEM- and SHV-derived {beta}-lactamases, they were divided into two subclasses, 2a and 2b(Bush, Jacoby and Medeiros, 1995).

a.GROUP 2a

Penicillinase, molecular class A The *2a subgroup* contains just penicillinases.

b.GROUP 2b

Broad-spectrum, molecular class A *2b* Opposite to 2a, 2b are broad-spectrum {beta}-lactamases, meaning that they are capable of inactivating penicillins and cephalosporins at the same rate. Furthermore, new subgroups were segregated from subgroup 2b:

2.3.5.1.3 GROUP 2be

Extended -spectrum, molecular class A *Subgroup 2be*, with the letter "e" for extended spectrum of activity, represents the ESBLs, which are capable of inactivating third-generation cephalosporins(ceftazidime, cefotaxime, and cefpodoxime) as well as monobactams(aztreonam).

2.3.5.1.4 GROUP 2br

Inhibitor-resistant, molecular class A *(diminished inhibition by clavulanic acid)*The *2br* enzymes, with the letter "r" denoting reduced binding to clavulanic acid and sulbactam, are also called inhibitor-resistant TEM-derivative enzymes; nevertheless, they are commonly still

susceptible to tazobactam,except where an amino acid replacement exists at position met69.

2.3.5.1.5 GROUP 2c

Carbenicillin, molecular class A Later *subgroup 2c* was segregated from group 2 because these enzymes inactivate carbenicillin more than benzylpenicillin, with some effect on cloxacillin.

2.3.5.1.6 GROUP 2d

Cloxacillanase, molecular class D or A *Subgroup 2d* enzymes inactivate cloxacillin more than benzylpenicillin, with some activity against carbenicillin; these enzymes are poorly inhibited by clavulanic acid, and some of them are ESBLs.The correct term is " Cloxacillanase ". These enzymes are able to inactivate the oxazolylpenicillins like oxacilli, cloxacilli, dicloxacillin. The enzymes belong to the molecular class D not molecular class A.

2.3.5.1.7 GROUP 2e

Cephalosporinase, molecular class A *Subgroup 2e* enzymes are cephalosporinases that can also hydrolyse monobactams, and they are inhibited by clavulanic acid.

2.3.5.1.8 GROUP 2f

Carbapenamase, molecular class A *Subgroup 2f* was added because these are serine-based carbapenemases, in contrast to the zinc-based carbapenemases included in group 3.

2.3.5.1.9 Group 3

Metallo enzyme,molecular class B *(not inhibited by clavulanic acid)*Group 3 are the zinc based or metallo {beta}-lactamases, corresponding to the molecular class B, which are the only enzymes acting by the metal ion zinc, as discussed above. Metallo B-lactamase is able to hydrolyse penicillins,cephalosporins, and carbapenems. Thus,

carbapenems are inhibited by both group 2f (serine-based mechanism) and group 3 (zinc-based mechanism)

2.3.5.1.10 Group 4

Penicillinase, no molecular class *(not inhibited by clavulanic acid)*

Group 4 are penicillinases that are not inhibited by clavulanic acid, and they do not yet have a corresponding molecular class.

2.3.5.2 Molecular Classification

The molecular classification of beta lactamases is based on the nucleotide and amino acid sequences in these enzymes. To date, four classes are recognised (A-),correlating with the functional classification .Classes A, C, and D act by a serine based mechanism, whereas class B or metallo beta lactamases need zinc for their action.

2.3.6 Genes Encoding ESBLs

In the early years the most common beta lactamases were TEM and SHV varieties (Pitout *et al*., 2010; Shobha *et al*.,2007; Florijn *et al*., 2002). TEM-2 and SHV-2 ESBL are derived from parental TEM-1 and SHV-1 by point mutation, which was mentioned before.TEM-1 and SHV-2 are non ESBL, but CTX-M enzymes are not derived from non ESBL and consequently all CTX-M enzymes are ESBL (Al-Agamy *et al*.,2009). Now, CTX-M enzymes are being discovered throughout the world and becoming the most prevalent beta lactamase (Xu *et al*.,2005).ESBLs (group 2be) are encoded on mutated TEM-1 and SHV-1 genes carried on plasmids that are readily transmissible to other organisms (Jemima and Verghese, 2008).

2.3.7 Extended Spectrum Beta Lactamase Types

2.3.7.1 TEM Beta-Lactamases (class A)

The TEM -1 enzyme was first reported from an *E.coli* isolate in 1965 and is now the commenest beta lactemase found in Enterobactereceae*(*Fonze

et al.,1995) and the older TEM is derived from Temoniera, a patient from whom the strain was first isolated in Greece(Turner,2005). TEM-1 is the most commonly-encountered betalactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. Also responsible for the ampicillin and penicillin n resistance that is seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers.Although TEM-type beta-lactamases are most often found in *E. coli* and *K.pneumoniae*, they are also found in other species of Gram-negative bacteria with increasing frequency. Based upon different combinations of changes, currently 195 TEM-type enzymes have been described. TEM-2,the first variant described, differed from TEM-1 through the substitution of a lysine for a glutamine at position 39 (Rupp *et al*.,2003). TEM and SHV are transferred by both plasmid and chromosome (Sharma,2010).TEM -3 most common in France (Livermore, 1995). TEM-10, TEM-12, TEM-3 and TEM -26 seem most common in the USA (Farkosh,2007).

The amino acid substitutions responsible for the ESBL phenotype cluster around the active site of the enzyme and change its configuration, allowing access to oxyimino-beta-lactam substrates. Opening the active site to beta-lactam substrates also typically enhances the susceptibility of the enzyme to β-lactamase inhibitors, such as clavulanic acid. Single amino acid substitutions at positions 104, 164, 238, and 240 produce the ESBL phenotype, but ESBLs with the broadest spectrum usually have more than a single amino acid substitution (Deepti Rawat and Deepthi Nair,2010 ; Paterson and Bonomo,2005).

2.3.7.2 SHV Beta-Lactamases (class A)

SHV-1 shares 68 percent of its amino acids with TEM-1 and has a similar overall structure. SHV stands for Sulf hydril variable (Turner,2005). The SHV-1 betalactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. ESBLs in this family also have amino acid changes around the active site, most commonly at positions 238 or 238 and 240. More than 60 SHV varieties are known. They are the predominant ESBL type in Europe and the United States and are found worldwide.SHV-5 and SHV-12 are among the most common (Farkosh, 2007). SHV varients are important worldwide **(**Rahman *et al*.,2004**)**. Among the SHV type of ß-lactamases,SHV-5 was found to be responsible for outbreaks of nosocomial infection in several countries (Jmima and Verghes,2008).

2.3.7.3 CTX-M Beta-Lactamases (class A)

Acquired resistance to beta-lactams is mainly mediated byESBLsthat confer bacterial resistance to all beta-lactams except carbapenems and cephamycins, which are inhibited by other beta lactamase inhibitors such as clavulanic acid. A shift in the distribution of different ESBLs has recently occurred in Europe, with a dramatic increase of CTX-M enzymes over TEM and SHV variants (Coque *et al*.,2008; Livermore and Canton,2007). CTXM β-lactamases (i.e. .active on CefoTaXime, first isolated in Munich.) were first reported from Japan in 1986 (the enzyme was initially named TOHO-1 and was later changed to CTX-M) (Matsumoto,1988). During the 1990s, general dissemination and occasional nosocomial outbreak, mostly of CTX-M-2-producing Enterobacteriaceae,were reported from South America (especially Argentina) (Peirano and Pitout,2010).

However, since 2000, *E.coli* producing CTX-M β-lactamases have emerged worldwide as an important cause of community-onset urinary tract infections (UTIs) and this has been called .the CTX-M pandemic.(GutkindandCátedrade,2001).

These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e.g., ceftazidime, ceftriaxone, or cefepime) (Pitout *et al*.,2008). Rather than arising by mutation, they represent examples of plasmid acquisition of beta-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms (Coque *et al*.,2008). These enzymes are not very closely related to TEM or SHV beta-lactamases in that they show only approximately 40% identity with these two commonly isolated beta-lactamases. The change at position 102 mainly enhances resistance to Ceftazidime, while the change at position 236 predominantly augments resistance to Cefotaxime, with a slight effect for Ceftazidime (Rahman *et al*.,2004). More than 80 CTX-M enzymes are curren.tly known, they are currently devided into 5 clusters on the basis of amino acid sequence: CTX-M-1,CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Al-Agamy *et al*., 2009; Smet *et al*.,2010) named after the enzyme first discovered for each lineage (Pagani et al., 2003). Despite their name, a few are more active on ceftazidime than cefotaxime. CTX-M-15 belongs to the CTX-M-1 cluster and is derived from CTX-M-3 by one amino acid substitution at position 240 (Asp→Gly); however, the flanking sequences of the -βlactamases can be very different (Peirano *et al*.,2010). The gene encoding CTX-M-14 differs from that encoding CTX-M-9 by only one amino acid change, at position 231 (Ala3Val),whereas CTX-M-13 differs from CTX-M-9 by four amino acid substitutions, at positions 3 (Val3Met), 53 (Val3Lys), 154 (Ala3Glu), and 231 (Ala3Val). Between CTX-M-13 and CTX-M-14,

there are three amino acid substitutions at positions 3, 53,and 154 (Chanawong *et al*.,2002).The initial observation of infections caused by bacteria harboring ESBLs in hospitals would suggest that CTX-M arose in the nosocomial setting and spread to the community (Perez *et al*., 2007). The epidemiology of organisms producing CTX-M enzymes is very different from those that produce TEM-derived and SHV-derived ESBLs (Pitout *et al*.,2008). Epidemiological reports demonstrate that some enzymes are more frequently reported than others, that predominant enzyme type varies with country and that diverse CTX-M types often exist within a single country (Ensor *et al*.,2006).CTX-M-15-producing *E. coli* are emerging worldwide, especially since 2003,as an important pathogen causing community-onset and hospital-acquired infections (Pirano *et al*., 2010). CTX-M enzymes (most often CTX-M-14 and -27) have been described in Asia especially since the late 1990s and early 2000s.Reports on the presence of CTX-M-15 in Asia remains relatively scarce outside of those studies from the subcontinent (i.e. India and Pakistan) (Hawkey *etal*.,2008). Reports from India indicate that *E. coli* producingCTX-M-15 is very common in the community as well as hospital settings (Ensor *et al*.,2006). India represents a significant reservoir and source of *E.coli* producing CTX-M-15 β-lactamases (Pirano *et al*.,2010).CTX-M-15 was found 73% in India in 2006 by (Ensor *et al.*, 2006).A strain that is thought to be responsible for the pandemic dissemination of the CTX-M-15 enzyme (Coque *et al*., 2008). CTX-M-15 have been reported from most countries in Europe ,Asia, Africa,North America, South America and Australia (Pirano *et al* .,2010). Group 9 (CTX-M-9 and 14) enzyme is dominant in Spain and Group 1 enzymes (particularly CTX-M 3 and CTX-M-15) is everywhere (Livermore,2007). The bowel is a rich environment for genetic exchange between commensal Enterobacteriaceae. Faecal carriage of CTX-M

producing bacteria has been described. It is plausible to suggest that conditions of overcrowding and poor sanitation, and the selective pressure created by overuse of antibiotics in India has enabled such widespread dispersal of CTX-M-15 (Ensor *et al*.,2007).

2.3.7.4 OXA Beta-Lactamases (class D)

OXA beta-lactamases were long recognized as a less common but also plasmidmediated beta-lactamase variety that could hydrolyze oxacillin and related antistaphylococcal penicillins. These beta-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The OXAtype beta-lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid. Amino acid substitutions in OXA enzymes can also give the ESBL phenotype (Wikipedia,2010).

2.3.7.5 AmpC-type- β-lactamases (Class C)

AmpC type β-lactamases are commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria. AmpC -β lactamases (also termed class C or group 1) are typically encoded on the chromosome of many Gram-negative bacteria including *Citrobacter*, *Serratia* and *Enterobacter* species where its expression is usually inducible; it may also occur on *E.coli* but is not usually inducible, although it can be hyperexpressed. AmpC type β-lactamases may also be carried on plasmids (Wikipedia2010).

2.3.7.6 Carbapenemases

Carbapenems are famously stable to AmpC -lβ-Lactamases and extended-spectrum-β-lactamases. Carbapenemases are a diverse group of b-lactamases that are active not only against the oxyimino-cephalosporins and cephamycins but also against the carbapenems(Wikipedia 2010).

2.3.7.7 Verona Integron-encoded Metallo- β-lactamase

A second growing family of carbapenemases, the VIM family, was reported from Italy in 1999 and now includes 10 members, which have a wide geographic distribution in Europe, South America, and the Far East and have been found in the United States. VIM-1 was discovered in *P.aeruginosa* in Italy in 1996 (Wikipedia 2010).

2.3.7.8 Oxacillinase group of β-lactamases class D

The OXA group of -β lactamases occurs mainly in Acinetobacter species and is divided into two clusters. OXA carbapenemases also tend to have a reduced hydrolytic efficiency towards penicillins and cephalosporins (Wikipedia 2010).

2.3.7.9 *K. pneumoniae* Carbapenemase class A

A few class A enzymes, most noted the plasmid-mediated KPC enzymes, are effective carbapenemases as well. Three variants are known, distinguished by one or two amino-acid substitutions. KPC-1 was found in North Carolina, KPC-2 in Baltimore and KPC-3 in New York. Plasmid -borne KPC enzymes are emerging among *K.pneumoniae* and other Enterobactericeae.The class A *Klebsiella pneumoniae*carbapenemase (KPC) is currently the most common carbapenemase, which was first detected in North Carolina, USA, in 1996 and has since spread worldwide.A later publication indicated that Enterobacteriaceae that produce KPC were becoming common in the United States (Rhee,2010).

2.3.7.10 New Delhi metallo-β-lactamase)

Originally described from New Delhi in 2009, this gene is now widespread in E.coli and *K.pneumoniae* from India and Pakistan. As of mid-2010,NDM-1 carrying bacteria have been introduced to other countries (including the USA and UK), presumably by medical tourists undergoing surgery in India. Inhibitor-Resistant β-Lactamases (Wikipedia 2010).

2.3.8 Important Facts Concerning ESBLs

2.3.8.1 Inhibitor-Resistant β-lactamases

Although the inhibitor-resistantβ -lactamases are not ESBLs, they are often discussed with ESBLs because they are also derivatives of the classical TEM- or SHV-type enzymes. These enzymes were at first given the designation IRT for inhibitor-resistant TEM -β lactamase; however, all have subsequently been renamed with numerical TEM designations. There are at least 19 distinct inhibitor-resistant TEM -β lactamases.These beta-lactamases have primarily been detected in France and a few other locations within Europe (Wikipedia 2010).

2.3.8.2 Organisms responsible for ESBLs

ESBLs are most common in *E.coli* and *K.pneumoniae* but do occure in other Enterobacteroceaespecially *Enterobacter, Proteus, Morganella morganii and Pseudomonas aeroginosa* (Shobha, 2007; Sasirekha, 2010; Peirano *et al*.,2010).Members of the bacterial family *Enterobacteriaceae* are found in the environment but also make up part of the normal microbiota of the intestine in humans and other animals. They are rod-shaped and stain Gram-negative, non-sporulating, facultative anaerobes that ferment different carbohydrates to obtain carbon(Farmer *et al* .,2007).They may grow as mucoid colonies when cultivated on agar plates, but only *Klebsiella* spp. are truly encapsulated (Abbott *et al*., 2007).The *Enterobacteriaceae*can be divided in 51 genera(Euzéby *et al*., 2011).and the number of species is continuously increasing.Members of the *Enterobacteriaceae* can cause many different kinds of infections.UTIs are the most common, followed by pneumonia, wound infections and infections of the bloodstream and central nervous system, see table 1. Some genera are common causes of intestinal infections such as enteritis and diarrhoea. They also make up an essential part of nosocomial infections, especially catheter related UTIs and ventilator associated

pneumonia ((Farmer *et al*.,2007; Abbott *et al*., 2007; Donnenberg *et al*., 2005 and Nataro ,2007).

Table 1. Clinically important members of the family Enterobacteriaceae commonly causing infections (Farmer *et al*., 2007; Abbott *et al* .,2007 ; Donnenberg *et al* .,2005 and Nataro ,2007).

Genus	Clinically important species	Common type of infections
Escherichia	*E. coli*	UTIs, diarrhoea, septicaemia, meningitis
Klebsiella	*K. pneumoniae, K. oxytoca*	UTIs, pneumonia, septicaemia
Proteu	*P. mirabilis, P. vulgaris*	UTIs, pneumonia, septicaemia, meningitis, wound infections
Enterobacter	*E. aerogenes, E. cloacae*	UTIs, pneumonia, septicaemia, wound infections
Citrobacter	*C. freundi*	UTIs, pneumonia, meningitis, septicaemia, wound infections
Salmonella	*S. enteritica*	diarrhoea, typhoid fever, septicaemia, UTIs, osteomyelitis
Shigella	*S. sonnei, S. flexneri*	diarrhoea, dysentery
Providencia	*P. rettgeri, P. stuartii*	UTIs
Morganella	*M. morganii*	UTIs, septicaemia
Serratia	*S. marcescens, S.liquefaciens*	UTIs, pneumonia, wound infections, septicaemia
Yersinia	*Y. pestis, Y. enterocolitica*	plague, enteritis, diarrhoea, septicaemia
Plesiomonas	*P. shigelloides*	diarrhoea, septicaemia

2.3.8.3 Risk factors for developing ESBL infection

Patients at high risk for developing colonization or infection with ESBL-producing organisms are often seriously ill patients with prolonged hospital stays and in whom invasive medical devices are present (urinary catheters, endotracheal tubes, central venous lines) for a prolonged duration (Deepti Rawat and Deepthi Nair,2010). In addition, other risk factors have been found in individual studies, including the presence of nasogastric tubes, gastrostomy or jejunostomy tubes or arterial lines; administration of total parenteral nutrition, recent surgery, hemodialysis, decubitus ulcers and poor nutritional status (Deepti Rawat and Deepthi Nair,2010).

Heavy antibiotic use is also a risk factor for acquisition of an ESBL-producing organism (Deepti Rawat and Deepthi Nair,2010). Several studies have found a relationship between third-generation cephalosporin use and acquisition of an ESBL-producing strain (Murki *et al*.,2010). However, perhaps the greatest risk factor for nosocomial acquisition of an ESBL-producing organism is accommodation in a ward or room with other patients with ESBL-producing organisms.Although there is no conclusive evidence, one potential source of colonization with the ESBL producers in the community may be the use of veterinary oxyimino cephalosporins like ceftiofur in livestock (Deepti Rawat and Deepthi Nair,2010).

2.3.8.4 Risk groups of Extended Spectrum Beta Lactamase

Patients at high-risk for ESBL include:

Neutropenic patients, transplant recipients, premature neonates, elderly persons and post-gastrointestinal surgery. Also high risk units inside hospitals include:Intensive care units, Hematology/oncology units,and Long term/ chronic care facility (Peirano*et al*.,2010; Farkosh,2007). Use

of extended spectrum antibiotics exerts a selective pressure for emergence of ESBL producing Gram negative rods (GNR) (Farkosh *et al*.,2007).

2.3.8.5 Extended Spectrum Beta Lactamase Carriage

Patients with known ESBL carriage should have their records flagged consistent with established policies. Upon readmission consider screening for ESBL. Sites most often sampled for carriage are those where the microorganisms are typically found -perianal/rectal and urine (Champs *et al*.,1989).

Patients with persistent carriage (e.g., 3 consecutive positive samples taken at least a week apart and the continuation of ESBL-associated risk factors) do not require continued screening during an admission. It is need to re-screen during the admission if there are changes in ESBL-associated risk factors. Re-screening should be determined on an individual patient basis. Factors to consider include: continuing use of antibiotics, predicted invasive interventions, or proposed removal of precautions (Friedman *et al.,* 2004; Shobha *et al*., 2007).During discharge the antibiotics should be informed about a patient.s ESBL-carriage as with any antimicrobial-resistant microorganism (Friedman *et al*.,2004).Most colonized patients are asymptomatic and may be a source of transmisssion to others (Friedman *et al*.,2004). The bowel is a rich environment for genetic exchange between commensa Enterobacteriaceae (Ensor *et al*., 2006).

2.3.8.6 Transmission

CTX-M gene mobilizes 10x more frequently than SHV & TEM gene (CLSI 2010).

The molecular epidemiology of ESBL outbreaks indicates that the mechanism of spread may be clonal strain dissemination, clonal plasmid dissemination and selection among polyclonal strains or both (Perez *et al*., 2007).The typical method of transmission includes clonal

dissemination of an ESBL producer strain or the dissemination of a plasmid carrying an ESBL gene (Coque *et al*.,2008). Selective antibiotic pressure then leads to colonization of patient.s bowel and skin with a risk of subsequent infection. Thus, fecal colonization may play a critical role in facilitating spread (Ensor *et al*.,2007). Outbreaks associated with procedures, e.g., catheterization,and contamination of medical devices has been reported (Pitout *et al*.,2008).Spread then appears to occur mainly through healthcare personnel hands. Endemic strains may persist in health care settings for years because of patient colonization,environmental contamination, and hand transmission (Friedman *et al*.,2004).Patient to patient transfer of microorganisms via the hands of healthcare workers is thought to be the main mode of transmission for ESBLs, although some ESBL outbreaks have been attributed to contaminated medical devices (e.g., ultrasound gel).Thus hand hygiene should be the most effective preventive measure (Friedman *et al.*, 2004).

2.3.8.7 Prevention and Control of Extended Spectrum Beta Lactamases

Proper infection-control practices and barriers are essential to prevent spreading and outbreaks of ESBL-producing bacteria. The reservoir for these bacteria seems to be the gastrointestinal tract of patients. Alternative reservoirs could be the oropharynx, colonized wounds and urine. The contaminated hands and stethoscopes of healthcare providers are important factors in spreading infection between patients (Deepti Rawat and Deepthi Nair,2010). Essential infection-control practices should include avoiding unnecessary use of invasive devices such as indwelling urinary catheters or IV lines, hand washing by hospital personnel, increased barrier precautions, and isolation of patients colonized or infected with ESBL producers.At an institutional level, practices that can minimize the spread of such organisms include clinical

and bacteriological surveillance of patients admitted to intensive care units and antibiotic cycling; as well as policies of restriction, especially on the empirical use of broad-spectrum antimicrobial agents such as the third- and fourth-generation cephalosporins and quinolones (Deepti Rawat and Deepthi Nair,2010).To combat overuse and misuse of antibiotics, the diagnosis of infectious diseases must be strengthened and antimicrobial resistance must be emphasized in education of health professionals and the general public.Hand hygiene is a simple and effective infection control intervention. Dirty or contaminated hands can transmit microorganisms which may cause infection .Hand washing with soap and water is effective (Friedman *et al*.,2004).Contact precautions in addition to other infection prevention measures, e.g., hand hygiene, environmental cleaning, and restriction of antibiotics, have been shown to be effective in preventing transmission in outbreak situations, use of gloves and aprons/gowns. No additional precautions are required in outpatient or home care settings (Friedman *et al*.,2004).Single (private) room is preferred. Spatial separation may be used. Cohorting of known cases, particularly in clusters/outbreaks, is acceptable. If there are limited single room accommodations in a facility or if sharing a room with a non-ESBL patient is required (e.g., long-term care facility, nursing homes, residential home),consideration should be given to the following: ensure the non-ESBL patient does not have risk factors, such as indwelling devices, neutropenia, history of transplantation,etc., and the non-ESBL patient has good hygiene practices (Friedman *et al*.,2004).

Monitoring and control of usage of extended spectrum cephalosporins and regular surveillance of antibiotic resistance patterns as well as efforts to decrease use as empirical therapy is indicated (Rupp *et al*.,2003).

2.3.9 Problems in ESBLs Detection

Identifying ESBL-producing organisms is a major challenge for the clinical microbiology laboratory. Multiple factors contribute to this, including production of multiple different β-lactamase types by a single bacterial isolate and the production of ESBLs by organisms that constitutively produce the AmpC β-lactamases, varying substrate affinities and the inoculum effect.The phenotypic confirmatory tests are highly sensitive and specific compared to genotypic confirmatory tests. However, there are a number of instances whereby the phenotypic confirmatory tests may be falsely positive or negative (Deepti Rawat and Deepthi Nair,2010).

K.pneumoniae or*E.coli* isolates which lack ESBLs but which hyperproduce SHV-1 may give false-positive confirmatory test results. Such isolates can have ceftazidime MICs as high as 32 μg/mL (Deepti Rawat and Deepthi Nair,2010).There are now numerous reports in which *K.pneumoniae* isolates have been found to harbor plasmid-mediated AmpC-type β-lactamases. Some of these organisms have been found to harbor both AmpC-type β-lactamases and ESBLs (Kohner *et al*.,2009).The coexistence of both enzyme types in the same strain not only results in elevated cephalosporin MICs but may also give false-negative test results for the detection of ESBLs. The likely explanation is that AmpC-type β-lactamases resist inhibition by clavulanate and hence obscure the synergistic effect of clavulanate and cephalosporins against ESBLs.For ESBL-producing bacteria, there is a dramatic rise of MIC for extended-spectrum cephalosporins as the inoculum is increased beyond that used in routine susceptibility tests. Same isolates test susceptible at the standard inoculum and resistant at a higher inoculum. Therefore, false-negative results can occur with both screening and confirmatory

tests when lower inocula are used (Deepti Rawat and Deepthi Nair,2010).Some ESBL isolates may appear susceptible to a third-generation cephalosporin in vitro, particularly if relatively high breakpoints are used. However, treatment of infections due to an ESBL-producing organism with third-generation cephalosporins may result in clinical failure even when the MIC is below the breakpoint and the ability of these enzymes to confer resistance to weak-substrate cephalosporins is clear when MIC determinations are performed with heavy inoculum. This may be due to the variable affinity of these enzymes for different substrates and inoculum effect.Many ESBL producers are resistant to combinations despite appearing sensitive *in vitro*. This could be due to hyperproduction so that the inhibitor is overwhelmed, relative impermeability of the host or co-production of inhibitor-resistant penicillanases (e.g., OXA-1).Since ESBL production is usually plasmid mediated, it is possible for one specimen to contain both ESBL-producing and non–ESBL-producing cells of the same species. This suggests that for optimal detection, several colonies must be tested from a primary culture plate (Deepti Rawat and Deepthi Nair,2010).ESBL enzymes can be induced by certain antibiotics, amino acids or body fluids. Organisms possessing genes for inducible β-lactamases show false susceptibility if tested in the uninduced state (Deepti Rawat and Deepthi Nair,2010).All these factors make detection of ESBLs a complicated and complex task, and improvements in the ability of clinical laboratories to detect ESBL are needed.Two opposing viewpoints have arisen in recognition of the poor outcome when patients with an infection due to an ESBL-producing organism are treated with a cephalosporin to which it appears susceptible *in vitro*. Some investigators believe that alteration of cephalosporin breakpoints for Enterobacteriaceae by organizations such as the Clinical and Laboratory Standards Institute is a more appropriate endeavor than

expanding efforts to detect ESBLs, which is too complex a task for a clinical microbiology laboratory. An advantage of such a change would be that organisms such as *Enterobacter* spp., which are not currently considered in CLSI guidelines for ESBL detection, would be covered.Another viewpoint is that the inoculum effect is important for ESBL-producing organisms. *In vitro*, the MICs of cephalosporins rise as the inoculum of ESBL-producing organisms increases (Deepti Rawat and Deepthi Nair,2010).Thus in the presence of high-inoculum infections (for example, intra-abdominal abscess, some cases of pneumonia) or infections at sites in which drug penetration may be poor (for example, meningitis, endocarditis or osteomyelitis), physicians should avoid cephalosporins if an ESBL-producing organism is present. Also severity of illness could have been greater in patients infected with organisms with higher MICs.A point favoring efforts aimed at ESBL detection is the infection control significance of detecting plasmid-mediated multi-drug resistance. There are epidemiologic implications for the detection of ESBL-producing organisms, as the significance of this resistance may not be as apparent if organisms are simply reported as intermediate or resistant to individual cephalosporins. Outbreaks of ESBL-producing organisms can be abruptly halted using appropriate infection-control interventions. Endemic transmission of ESBL producers can also be curtailed using infection-control measures and antibiotic management interventions. Detection of ESBL production in organisms from samples such as urine may be important because this represents an epidemiologic marker of colonization (and therefore the potential for transfer of such organisms to other patients).

2.3.10 ESBLs Treatment Options

2.3.10.1 Carbapenem

The carbapenem antibiotic family includes biapenem, doripenem, ertapenem, faropenem (a closely related penem to carbapenems), imipenem, meropenem, panipenem, thienamycin and razupenem. These penems originally developed from thienamycin, a natural, fungal-derived product of Streptomyces cattleya(Kahan*etal.*, 1979).In this situation, carbapenem antibiotics remain the drug of choice and have been increasingly utilized to treat infections with ESBL-producing organisms(Pitout, 2010).Carbapenem(imipenem,meropenem,ertapenem) choice of drug for treating the ESBL producing bacteria (Samaha-Kfoury and Araj,2003).

2.3.10.2 Quinolones

Fluoroquinolones have been considered as attractive alternatives inthe treatment of infections due to ESBL-producingorganisms. Unfortunately, there is an increasingassociation between ESBL production and fluoroquinoloneresistance(Patterson*etal*., 2000) **.**

If there is in vitro susceptible to ciprofloxacin, a satisfactory clinical response can be achieved by using quinolones (Samaha-Kfoury and Araj,2003).

2.3.10.3 Aminoglycosides

As in the case with quinolones, aminoglycosides are effective therapy against ESBL producing pathogens .Susceptibility to amikacin seems to be preserved, in contrast to gentamicin and tobramycin, thus justifying its use as empiric therapy (Samaha-Kfoury and Araj,2003).

2.3.10.4 Tigecycline

Tigecycline, a novel, first in class glycycline and an analogue of the semisynthetic antibiotic minocycline, is a potent, broad spectrum antibiotic that acts by inhibition of protein translation in bacteria by

binding to the 30S ribosomal subunit and blocking the entry of amino-acyl to RNA molecules into the A site of the ribosome (Amaya *et al* .,2009).CLSI criteria to interpret susceptibility testing of tigecycline are not yet established. *In vitro* data supports the notion that tigecycline can be considered an alternative to carbapenems for treatment of infections due to ESBL-producing

Enterobacteriaceae. However, clinical experience with tigecycline is still evolving (Rupp and Fey,2003).

2.3.10.5 Fosfomycin

Fosfomycin tromethamine is a soluble salt of fosfomycin with improved bioavailability over fosfomycin. It inactivates the enzyme

pyruvyltransferase , which is required for the synthesis of the bacterial cell wall peptidoglycan (Amaya *et al*.,2009). The excellent *in vitro* activity of fosfomycin against ESBL-producing *E.coli* and *K. pneumoniae* strains has been recently reported. Further studies are required to assess the efficacy of fosfomycin for the treatment of UTIs caused by ESBLproducing enterobacteria (Rupp and Fey,2003).

2.3.10.6 Colistin

Although once considered a toxic antibiotic, clinicians have now turned to colistin as a last resort agent for the treatment of infections caused by multidrug resistant gramnegative bacteria, against which this cationic detergent-like compound remains active.The antimicrobial target of colistin is the bacterial cell membrane,where the polycationic peptide ring interacts with the lipid A of Lipopolysacharides, allowing penetration through the outer membrane by displacing Ca + and Mg + . Insertion between the phospholipids of the cytoplasomic membrane leads to loss of membrane integrity and to bacterial cell death (Amaya *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

A Cross sectional laboratory based study was conducted to investigate and report ESBLs producers among population in a math gatherin area like Almadenah Almonawarah.

3.1.1 Study Area

Different Hospitals and clinical centers in Almadinah almonawrah region were included in the study.

3.1.2 Study Population and Duration

Different clinical specimens (n = 359) were collected from patients with signs and symptoms of bacterial infection, who attended to microbiology laboratory at Prince Sultan Military Hospital and King Fahad Hospital, Al Madinah Al-Monawarah during the period from January 2014 to August 2015. Patients were included in this study after being given their informed consents.

3.1.3 Data Collection

Basic data were collected by using a standard data questionnaire consisting of basic demographic data. Additional information were also included social status, history of previous infection, and history of using antibiotics (Appendix I) and from the electronic patient file by using OASIS electronic system.

3.1.4 Data Analysis

Data were analyzed using the SPSS statistical software package, Version 16 . Chi-square test was used to recognize differences within the data. p-values < 0.05 were considered as statistically significant.

3.2. Isolation and Identification ofEnterobacteriacea

3.2.1 Samples Collection

Atotal of 359 different clinical samples were collected frommost admitted samples included; urine, stool, body fluids sputum, pus blood, wound, high vaginal, ear and eyes swabs, tips and invasive medical devices were obtained from patients with symptoms of bacterial infection. Samples swabs obtained were collected by using sterile tipped swabs with transport media, sterile blood culture bottles from BD Company, sterile urine container direct aspiration of pus from deep-seated wounds. The samples were delivered to microbiology laboratory within two hours if delayedthey were stored in refrigerator at 2-8°C.

3.2.2 Inoculation of the specimens

All sampls were routinely cultured on MacConkey agar with crystal violet, CLED agar,XLD agar and blood agar plates.These plates were routinely incubated at 37°C aerobically for urine samples,stool and aerobically with 5% CO_2 forother samples, after overnight incubation.Then they were checked for bacterial growth.

3.2.3 Media and Culture Conditions

3.2.3.1 Blood Agar

All Enterobacteriaceae isolates were inoculated on blood agar (Oxoid media) and plates were incubated at 37°C for 24–48 hours.

3.2.3.2 MaCkonkey Agar

All Enterobacteriaceae isolates were inoculated on MacConkey agar (Oxoid) and were incubated at 37°C° for 24–48 hours.

3.2.3.3 Cystine lactose Electrolyte Deficient medium

All *Enterobacteriaceae* isolates were inoculated on CLED (Oxoid)and plates were incubated at 37°C for 24–48 hours aerobically.

3.2.3.4 Xylose Lysine Deoxycholate Agar

All stool samples were inoculated on XLD(Oxoid) and plates were incubated at 37°C for 24–48 hours aerobically.

3.2.3.5 Nutrient Agar plate

AllEnterobacteriaceae isolates were subcultured on nutrient agar (Oxoid) and incubated at 37°C for 24–48 hours for biochemical tests.

3.2.3.6 Maintenance and Preservation of Culture Strains

All Enterobacteriaceae weregrown in appropriate media for 18-24 hours were preserved in a nutrient agar slant at 2-8°Cin a refrigerator and this culture was used within two weeks for routine laboratory works. For long term preservation, strains were stored in brain heart infusion broth with 20% glycerol and stored frozen without significant loss of viability at -20°C until further study (Cheesbrough, 2006).

3.2.4 Gram's Stain

A drop of normal saline was taken on the centre of a clear glass slide and a colony was taken by a sterilized inoculating loop to make a thin emulsion. A thin film was prepared by spreading the emulsion uniformly. This film was fixed by passing it over the flame for two or three times. Smear was covered with crystal violet stain for 30 -60 seconds. Then stain was washed with distilled water and cover with Lugol.s iodine for 30-60- seconds. Again stain was washed with distilled water and decolourize with acetone alcohol and washed with distilled water .The back of the slide was wiped and placed it in a draining rack, for the smear to air dry.Tested specimen was examined and compared with positive(*S.areus* ATCC 29213) and negative control*(E.coli* ATCC 25922) under microscope.

3.2.5 Biochemical Tests

3.2.5.1 Spot Oxidase test (Cytochrome oxidase)

This test was used to identify the bacteria which produce the enzyme oxidase.Astrip of filter paper was impregnated with 1% w/v aqueous tetra methyl p-henylenediamin dihydrochloride solution (Appendix III). A speck of culture from the primary plate wasimmediately rubbed on it with a sterile loop. A positive reaction was indicated by adeep purple blue within 5-10 seconds (Cheesbrough, 2006).

3.2.5.2 Motility test

Motility test mediumwas used to test the motility of the bacteria. Semisolid media (0.5%) was used for this purpose. The organism was inoculated by stabbing a straight wire carrying the inoculums once vertically into the center of the agar butt.After overnight incubation, motility was shown by a spreading turbidity from the stab line or turbidity through out the medium (Cheesbrough, 2006).

3.2.5.3 Citrate utilization test

Simon citrate agar media(Oxoid) was used for differentiating the intestinal bacteria on the basis of citrate utilization. Colonies were picked with a straight wire and the organism was inoculated to the surface of Simon citrate agar slant. Citrate utilization was followed by alkaline reaction e.g., change of color from light green to blue (Cheesbrough, 2006).

3.2.5.4 Indole ProductionTest

Indole production was tested for some bacteria, which has the ability to degrade tryptophanto indole. Indole production was detected by Kovac reagent (4-dimethyl amino benzaldehyde, isoamyl alcohol, hydrochloric acid). The test organism was cultured in peptone broth which contains tryptophan. This broth was incubated at 37°Cfor overnight and Kovac

reagent was added. Development of red colour ring over the surface in the broth within 10 minutes indicates positive test (Cheesbrough, 2006).

3.2.5.5 Kligler Iron Agar(KIA)

Kligler Iron Agar was used for the differentiation of microorganisms on the basis of dextrose and lactose fermentation and hydrogen sulfide production. Using sterile straight loop The colonies were touched and inoculated on Kligler iron agar (HIMEDIA, India) and then incubated at 37°C overnight incubation (Cheesbrough, 2006) **.**

3.2.5.6 Urea Hydrolysis Test (ChristensenMethod)

This test was used to determine the ability of an organism to produce the enzyme urease, which hydrolyzes urea.Hydrolysis of urea, produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift isdetected by the color change of phenol red from light orange at pH 6.8 to magenta at pH 8.1.The surface of a urea agar slantwas streaked with a portion of a well-isolated colonies.the cap was left on loosely and the tube was incubated at 37°C in ambient air for 48 hours(Cheesbrough, 2006).

3.2.6 Full Automation Technique (BD PHOENIX 100)

3.2.6.1 Introduction

The BD phoenix (Automated microbiology system) (Horstkotte *et al.*, 2004) (Figure 4) was intended for the rapid identification (45 wells with integrated conventional biochemical tests substrates) and antimicrobial susceptibility testing(85 wells for antibiotics)) of clinically significant bacteria. The phoenix system provides rapid result for aerobic and facultative anaerobic .Gram-positive bacteria as well as most aerobic and facultative anaerobic gram-negative bacteria of human origin.

3.2.6.2 Materials Required

BD Phoenix automated microbiology system it should be supplemented with all these accessories: Phoenix ID Broth, Phoenix AST Broth,

Phoenix AST indicator solution ,Phoenix Gram negative Panels,Phoenix Inoculation Station,Phoenix Transportation Caddy,BBL™ Crystal Spec™ or BD Phoenix Spec™ Nephelometer,25µL pipettor (Figure 5).

Figure 4. BD Phoenix 100**,automated system for the identification and susceptibility testing of clinically relevant bacteria** from Bicton Dickinson company, US.

Figure 5. BD Phonix 100 accessories; A: ID broth, AST and the indicator solution; B: Phoenix gram negative Panels in side and out side pouch;C: BBL™ Crystal Spec™ or BD Phoenix Spec™ Nephelometer:D:Phoenix Inoculation Station; E:Caddy; F: 25µL pipettor.

3.2.6.3.Test Procedure for Identification of the Isolates

Once the Gram's stain reaction was confirmed, the appropriate Phoenix panel was selected for inoculation**.**The pouch of the panel was examined and the panel was removed from the pouch.The panel was placed on the inoculation station with the inoculation ports on top and the pad on the bottom.By using aseptic technique Phoenix ID broth tube was labeled with patient' specimen number.With the tip of sterile cotton swab or awooden applicator stick, the colonies of the same morphology was picked from the one of the recommended media and the colonies were put into the Phoenix ID(4.5 ml) and suspended.The tube was capped ,vortexed for five seconds and allowed approximately 10 seconds for air bubbles to surface.The tube was inserted in to the Crystal Spec or BD Phoenix Spec nephelometer for reading inoculums density (Carroll *et al*., 2006). The inoculums density was setted to 0.5 McFarland for the panel type was being run then a range of 0.50-0.60 is acceptable.

Note: The standardized bacterial suspension in ID broth must be used within 60 minutes of preparation (Brigante *et al*., 2006).Phoenix Antimicrobial Sensitivity Test(AST) broth tube (8.5 ml) was labeled with patient's specimen number and one free-falling drop of AST Indicator solutionto the AST broth tube was added and inverted to mix. 25µl of the bacterial suspension was transferred from the ID broth tube to the AST broth tube. The AST tube was capped and inverted several to mix and wait afew seconds for air bubbles to surface.The ID tube inoculum was poured in to the fill port on ID side of the panel (51-wells side for biochemical tests).The fluid was allowed to traverse down the tracks before moving the panel.The AST broth ionculum was poured in to the fill port on AST side of the panel (85-wells side of antimicrobial agents) , the fluid was allowed to traverse down the tracks before moving the panel , the residual drplets of inoculum on the edge of the fill port was

checked before placing panel closure,the panel closure was snapped, surely was fully seated and the panel was inspected visually to be sure each of the wells were full.The panels were logged into the instrumen for incubation at 37c□ . The time needed to obtain a complete set of Identification(ID) and Antimicrobial Sensitivity Test(AST) results varies between 6 and 12 hour and is dependent on the bacteria tested (Hirakata *et al*., 2005**).**All these control strains were run during the processing of the procedure,*E.coli* ATCC 25922,*P.aeruginosae* ATCC27853,*E.coli* ATCC 35218 and *K.pneumonia*e ATCC 700603.

3.3 Antibiotic test

All the isolated bacteria were tested in vitro for their antimicrobial resistance to various antibiotics by the Kirby-Baur disc diffusion and BDPhoenix 100 method.The antibiotiswhich were included for gram negative Enterobacteriacae were ceftazidime (30 µg) (CAZ);Cefotaxime (30 µg) (CTX); Norfloxacin (10 µg) (NOR); Amikacin (30 µg) (AK); Ceftriaxone (30 µg) (CRO); Co-trimoxazole (1.25/23.75 µg) (SXT); Nitrofurantoin (300µg) (NF); Cefoxitin (30 µg) (FOX); Pipracillin (100 µg) (PIP); Cefepime(30 µg) (FEP); Pipracillin/Tazobactam (100/10); Amoxyclav (20/10)(AMC); Imipenem (10 µg) (IPM) ;Tetraccycline (30 µg) (TE); Ampicillin (AM); Levofloxacin (LVX); Gentamicin (GM) and Cephalothin (CF). The sensitivity test was also performed on Muller-Hinton agar plates (pH 7.2-7.4). The surface was lightly and uniformly inoculated by sterile cotton swab stick. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards. The swab stick was then took out and squeezed on the wall of the test tube to discard extra suspension. Inoculated plates were incubated at 37°C degree for 24 hours. On the next day, plates were read by taking measurement of zone of inhibition. Inhibition zones were measured in millimeter (mm) by using a ruler over

the surface of the plate with the lid open. Plates were held a few inches above a black, nonreflecting background and illuminated with reflected light. Results were recorded and graded as resistant (R), intermediate (I) or sensitive (S), according to the reference zone of inhibition of particular antibiotic (NCCLS, 2001).

3.4 ESBLS Detection Methods

3.4.1 Phenotypic Methods

3.4.1.1 Disk Diffusion Test (ESBLs screening test)

The isolates were tested for their susceptibility to the third generation cephalosporins ceftazidime (30 µg), cefotaxime (30 µg) and ceftriaxone (30 µg) and aztreonam (30 µg) by using the standard disc diffusion method, as was recommended by the CLSI (CLSI, 2010). ESBL were screened by detection reduced zones of inhibition around third generation cephalosporins.The strain was considered to be "suspicious for ESBL production" (CLSI, 2010) If zone diameter of ceftazidime, < 22 mm, cefotaxime, < 27 mm, ceftriaxone, < 25 mm and aztreonam, < 27 mm. Only those isolates which were resistant to one of the third generation cephalosporins were selected for the study and they were processed for the ESBL production.

3.4.1.2 Phenotypic Confirmatory Test with Combination Disk

A third-generation cephalosporin antibiotic disk alone and in combination with clavulanic acid was used. In this study, two combinations were used,firstly a disk of Ceftazidime (30µg) alone and a disk of Ceftazidime + Clavulanic acid (30 µg/10 µg) and secondly Cefotexime(30µg) alone and a disc of Cefotaxime+Clavulanic acid (30µg/10µg). Both disks were placed at least 25 mm apart, center to center, on a lawn culture of the test isolate on Mueller Hinton Agar (MHA) plate and incubated overnight at 37°C. Difference in zone diameters with and without clavulanic acid was measured. Interpretation: When there was an increase of ≥ 5 mm in

inhibition zone diameter around combination disk of Ceftazidime + Clavulanic acid versus the inhibition zone diameter around Ceftazidime disk alone, it confirms ESBL production. *E. coli* ATCC 25922 was used as control negative and *K.pneumoniae* ATCC 700603 as control positive (CLSI, 2002).

3.4.1.3 Modified Double Disk Synergy Test (confirmatory test)

The ESBL production was tested by the Modified Double Disc Synergy Test (MDDST) by using a disc of amoxicillin-clavulanate (20/10 μg) along with fourcephalosporins; Third generation cephalosporin-cefotaxime, ceftriaxone, cefpodoxime and fourth generation cephalosporin -cefepime. A lawn culture of the organisms was made on a Mueller-Hinton agar plate, as was recommended by CLSI (2009). A disc which contained amoxicillin-clavulanate (20/10 μg) was placed in the centre of the plate. The discs of third generation cephalosporin and fourth generation cephalosporin were placed 15mm and 20mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc (Paterson DL and Bonomo RA,2005). Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production.*K.pneumoniae* ATCC 700603 was used as a control strain for a positive ESBL production and *E.coli* 25922 was used as a negative control for the ESBL production.

3.4.1.4 BD Phoenix 100 Automated System Method

The Phoenix ESBL test was used growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulanic acid, to detect the production of ESBLs The test algorithm has been delineated by (Sanguinetti *et al,*. 2003).Results were usually available within 6 hours and recommended Quality control strains were simultaneously tested with a non–ESBL-producing organism (*E.coli* ATCC 25922) and

an ESBL-producing organism (*K.pneumoniae* ATCC 700603) (Wayne, 2009).

3.4.2 Molecular Method

3.4.2.1 Polymerase Chain Reaction

3.4.2.1.1 Extraction of Bacterial DNA

Bacterial DNA was isolated from all pure ESBLs strains producers using ready kits(Thermo Fisher Scientific GeneJET Genomic, DNA purification kit). Purity of the isolated DNA was monitored by NanoDropper 2000 (Thermo SCIENTIFIC, USA).Genomic DNA templates for PCR amplification were gained from overnight growth of bacterial isolates on nutrient agar suspended in 500 µl of sterile deionized water, and boiled for 10 minutes. After centrifugation of the boiled samples at 14000 g for 10 minutes by Microcentrifuge (Appendix V), supernatant was stored at 20°C as a template DNA stock (Kazuki*et al*., 2014). The purity of the extracted DNA was determined by running the DNA sample on 2% gel agarose (Sambrook *et al*., 1989).

3.4.2.1.2 Multiplex PCR assay

PCR assay searching for *bla*TEM, *bla*SHV, and *bla*CTX-M genes among the 85 ESBLs positive isolates, was adopted as described by Karmele *et al*., (2003) with minor modification.A 25 µL reaction mixtures was prepared which contained 12.5 µL 2x GoTaq Green Master Mix PCR buffer (Promega, USA), 0.2 µL of each primer and 10.3 µL nuclease free water. Then 2 µL template DNA was added separately to each reaction tube with a final volume of 25 µL / Reaction. The PCR primers which were used in this study were listed in (Table 2).

3.4.2.1.3 PCR Amplification Program

The cycling conditions were as follows: denaturation at 94 °C for 10 min, 30 cycles of denaturation at 94 °C for 1 min, followed by

annealing at 60 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 7 min. A previously identified *K. pneumoniae* ESBL-positive isolate was used as a positive control, and a negative control (nuclease-free water) was included in each run (Meyer et al., 2007)..The amplification was done using CLASSIC K960 China thermal cycler (Appendix V).

3.4.2.1.4 Visualization of PCR Products

PCR products were visualized on 2% agarose gel using 1X TBE buffer which contains 0.5µg/ml ethedium promide and finally the PCR products were photographed under UV lamb (SYNGENE, UK) and bands sizes were compared to a 100 bp DNA ladder and it was done as follow:The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer which contais 0.5µg/ml ethedium promide just to cover the gel surface, 5 µl of PCR products from each samples was added to wells of electrophoreses, 5 µl of DNA ladder (100-bp DNA ladder, iNtRON, Korea) was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Primer, 100 V, 500 mA, UK) (Appendix V).The electrophoresis was carried out at 75 Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec – UK) (Appendix V).

Table 2. Target genes, sequences of primers and ampilicons size

Genes	Primer	Sequences	Ampilico Size	Refrences
BlaSHV	SHV-F	5'AGGATTGACTGCCTTTTTG 3'	392	K. Colm *ea al.*,(2003).
	SHV-R	5'ATTTGCTGATTTCGCTCG3'		
BlaTEM	TEM-F	5'ATCAGCAATAAACCAGC3'	516	K. Colm *ea al.*,(2003).
	TEM-R	5'CCCCGAAGAACGTTTTC3'		
BlaCTXM	CTX-M- F	5'TTTGCGATGTGCAGTACCAGTAA3'	544	M. Edelstein *et al.*,(2003)
	CTX-M -R	5'CGATATCGTTGGTGGTGCCATA3'		

CHAPTER FOUR

RESULTS

4.1 Epidemiological Findings

4.1.1 Demographic Data for Enrolled Participants

4.1.1.1 Collection of Specimens

Different clinical specimens (n = 359) were collected from patients with signs and symptoms of bacterial infection, during the period from January 2014 to August 2015. In relation to demographic and personal data,25 % of the participants were males while the remainder75% were females.

4.1.1.2Enrolled Patients versus Age Groups

Among the study of population when infected cases with ESBL(85) were grouped in to four age groups: age group one; less than 20 years old 19(22.4%); age group two (20-39) years old 21(24.7%) ;age group three40-59 years old 13(15.3%) ; age group four (more than 60 years old) 32(37.6%).The data showed that the frequency of distribution was greater in the age group four of more than 60 years old (Table 3).

Table 3 . Distribution of age groups among enrolled ESBL positivepatients.

Age group	Infected cases	%
Less than 20 years old	19	22.4
20-39 years old	21	24.7
40-59 years old	13	15.3
More than 60 years old	32	37.6
Total	85	100%

4.1.1.3 Antibiotic Treatment among Enrolled ESBL Positive Participants

In relation to frequency of antibiotics treatment against positive ESBL participants (85),were more inpatients under antibiotics treatment (71) 83.5% than those were not under treatment (14) 16.5%and also the frequency of females (51/85) 60% were more than males (34/85) 40% (Figure 6).

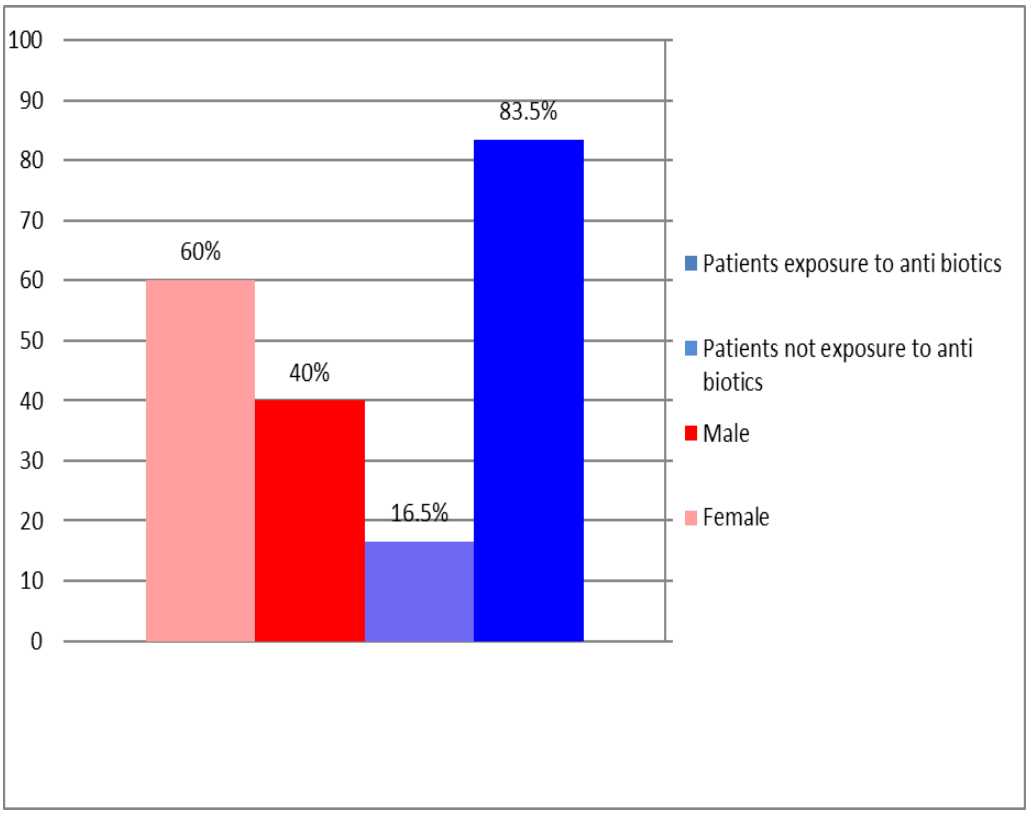

Figure 6. Frequency of antibiotics treatment against ESBL positive participants .

4.2 Bacteriological Findings.

4.2.1 Identification of the Isolates

The bacterial isolates obtained in this study were identified according to their cultural characteristic, colonial morphology, Gram reaction and their

biochemical properties.The result of these tests were listed in a colour plates (Appendix VI)and full automation technique (Phoenix 100).

Results reflected high burden of *E. coli* (189, 52.6%) followed by *K. pnumoniae* (87, 24.2%),proteus (23,6.4%), *Acinitobacter* spp (19 ,5.3 %) , *Providencia* spp (12 ,3.4 %), *Enterobacter cloacae* (12 ,3.4 %) , *Serretia marrcescenc* (4,1.1%), *Citrobacter* spp (3 ,0.8%) , *Sallmonella* spp (3, 0.8 %) and other (7, 2 %). The frequency of the different Enterobacteriaceaeisolated from study subjects is shown in Table 4.

Table 4. Frequency of different isolates among study subjects**.**

Bacterial Isolates	Total	%
Escherichia coli	189	52.6
Klebsiella pneumonia	87	24.2
Proteus spp.	23	6.4
Acinitobacter spp	19	5.3
Enterobacter cloacae	12	3.4
Providencia spp	12	3.4
Serretia marrcescenc	4	1.1
Citrobacter spp	3	0.8
Sallmonella spp	3	0.8
Others	7	2
Total	359	100

4.2.2 Antimicrobial Susceptibility Testing

Further-more, all ESBL and non-ESBL producers were tested against different empirical antibiotics,by Kirby-Bauerand BD Phoeinx 100 the results reflected significantly high rate of resistance among ESBL isolates compared with non-ESBL producers ($P < 0.05$) as shown in Table 5.

Table 5. Antimicrobial Susceptibility Pattern of ESBL and non-ESBL Producers

No	Antibiotics	ESBL-Producers		NonESBL-Producers		P value
		No	%	No	%	
1	Amikacin	28	32.9	23	8.4	< 0.05
2	Ampicillin	81	95.3	143	52.2	< 0.05
3	Aztereonam	81	95.3	25	9.2	< 0.05
4	Cephalothin	81	95.3	94	34.3	< 0.05
5	Ciprofoxacin	62	72.9	55	20.1	< 0.05
6	Gentamicin	21	24.7	50	18.3	< 0.05
7	Levofloxacin	51	60.0	52	19.0	< 0.05
8	Norfloxacin	58	68.2	51	18.6	< 0.05
9	Trimethoprime-Sulfamethaxazole	61	71.8	82	30.0	< 0.05

4.3 Detection of ESBL Producers

4.3.1 Phenotypic Detection of ESBLs

4.3.1.1 Disc Diffusion Screening Test

Isolates showing an inhibition zone size of ≤22 mm with ceftazidime (30 μg), ≤25 mm with cefriaxone (30 μg) and ≤27 mm with cefotaxime (30 μg) were identified as potential ESBL producers and were short listed for confirmation of ESBL production.

4.3.1.2 Confirmatory Combination Disc Diffusion Test

Both cefotaxime and ceftazidime,alone and in combination with clavulanic acid was performed by confirmatory combination disc diffusion testing and the result showed that a ≥ 5mm increase in azone diameter for antimicrobial agent tested in combination with calvulanic

acid (10mg) versus its zone when tested alone which was confirmed as ESBL-producing organisms (Figure7).

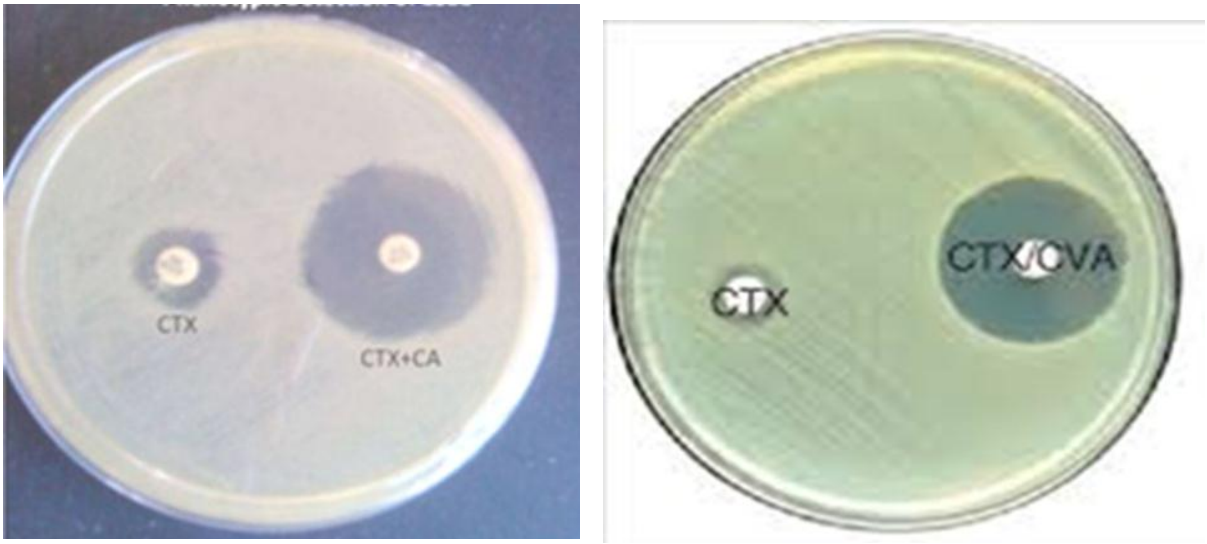

Figure 7 .Combination disc diffusion tests for phenotypic detection of ESBL in *E.coli* (left) and k.pnemoniae (Right).

4.3.1.3 ModifiedDouble Disc Synergy Test

All bacterial isolates were studied for ESBL production by the modified double disc test (MDDST)using third generation cephalosporins-cefotaxime (30 mg), ceftriaxone (30 mg), cefpopdoxime 30 mg and fourth generation cephalosporin- cefepime(30 mg)along with a amoxicillin-clavulanate (20/10 μg).The resultdiscswere showed enhancement of the zone of inhibition around one or more of the cephalosporins discs towards the disc of amoxicillin-clavulanate was indicative of ESBL production(Figure 8).

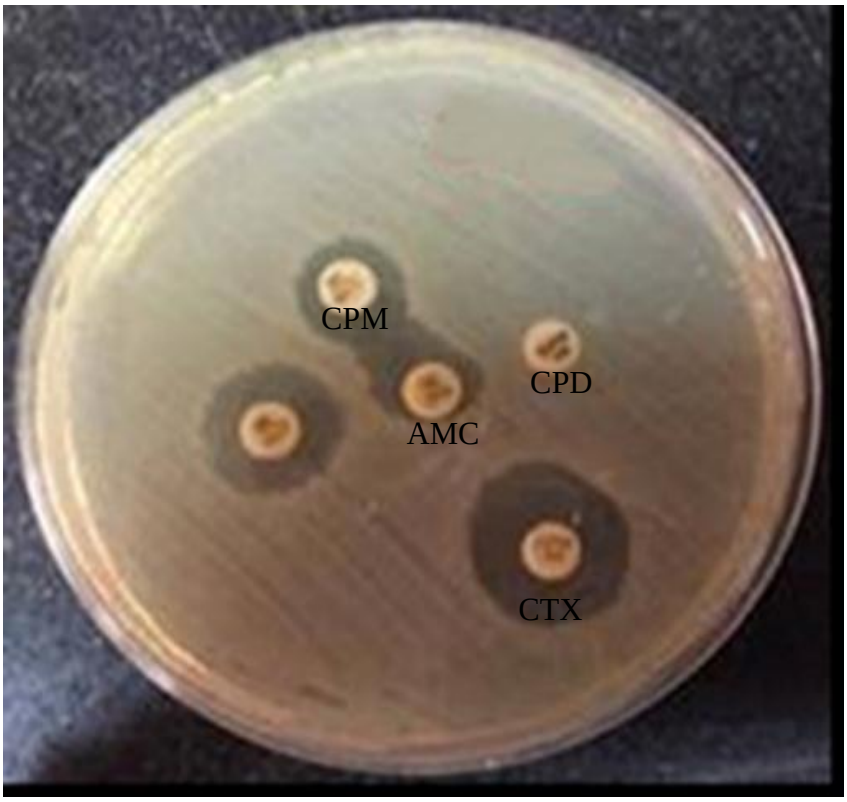

Figure 8. Mueller-Hinton agar plate showing phenotypic detection of ESBL production using the Modified double disk synergy test (MDDST) method,showing enhancement of zone inhibition of only cefepime but none of the third generation cephalosporin used with amoxicillin-clavulanate. (AMC–Amoxyclav, , CTX-Cefotaxime, CPD-Cefpodoxime, CPM-cefipime.

4.3.1.4 Full Automated System (BD Phoenix 100).

BD Phoenix was used for detection of ESBLS.

4.3.2 Frequency of ESBL Phenotype Producers

Of the total 359 bacterial isolated, 85 (23.7%) strains were defined as ESBLs and 274 (76.3%) were defined as non-ESBLs. Among the total 85 ESBL, *E.coli*was found to be the most frequent (66/77.6%), while *K. pneumonia* was (19/22.4%), (Figure 9).

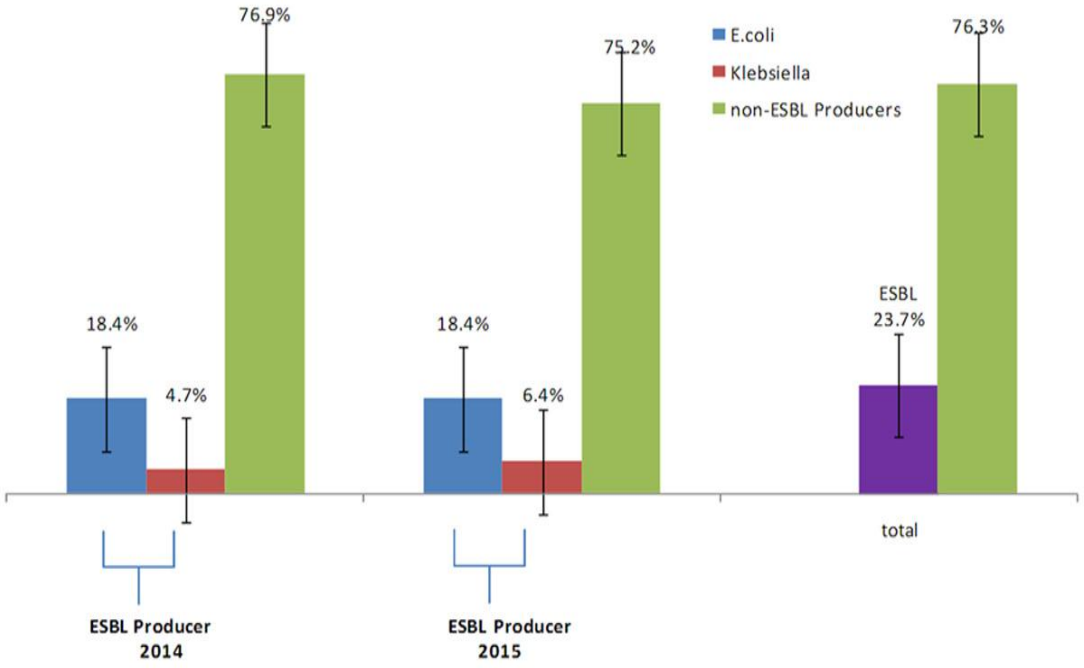

Figure 9. Distribution of ESBL isolates during the study period.

4.3.3Distribution of ESBL among Different Clinical Samples

Extended spectrum β-lactamases producers (ESBLs) were more frequent in urine specimens 221 (61.7%), followed by blood 54 (15.1%), pus 29 (8.2%), wound 20 (5.5%), sputum 15 (4.1%), eye 10 (2.7%) and high vaginal swap 10 (2.7%) as illustrated in(Figure 10).

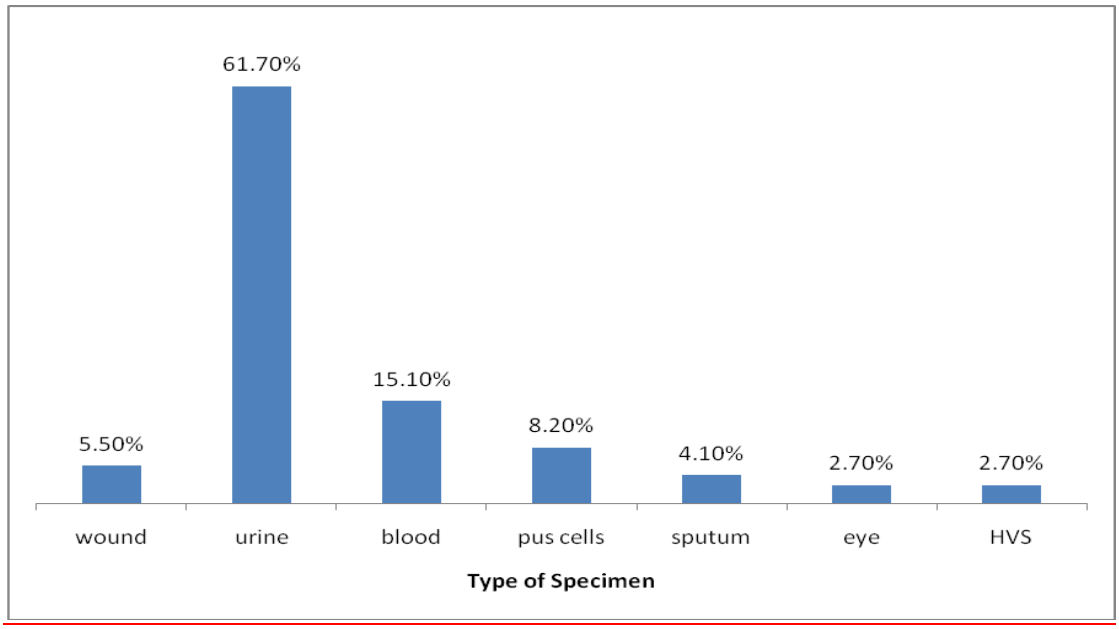

Figure 10. Distribution of ESBL among different clinical samples among enrolled subjects.

4.3.4 Distribution of ESBL in Different Hospital departments.

Our results also showed that, the ESBL-producing strains were most commonly isolated from ICU 26 (30.6 %) followed by Urology 21(24.7 %) the medium frequency was reported in family medicine and surgery department (11.7%). While the lowest was in Obstetrics and gynecology ,Pediatric, Respiratory, Internal medicine, Heamodialysis,Neonate with percentages less than 3% (Table 6).

Table 6. Distribution of ESBL in different hospital departments.

Departments	No of ESBL positive patients	%
ICU	26	30.6
Urology	21	24.7
Family medicine	10	11.7
Surgery	9	10.6
Obstetrics and gynecology	6	7
Pediatric	3	3.5
Respiratory	3	3.5
Internal medicine	2	2.4
Heamodialysis	2	2.4
NICU	2	2.4
VIP	1	1.2

4.3.2 Molecular Findings

4.3.2.1 Extraction of Genomic DNA

Bacterial DNA was extracted from all pure ESBLs producer isolates using ready kits from Thermo Scientific GeneJET Genomic, Leithuania. Purity of the isolated DNA was monitored by NanoDropper 2000 (Thermo SCIENTIFIC, USA).

4.3.2.2 Multiplex PCR in the detection of ESBL genes

All of the 85 isolates that were phenotypically flagged as ESBL producers were genotyped (Figures 11, 12). The results were as follows: 63 isolates (74.1%) possessed the CT-XM gene, 27 (31.8%) of the isolates possessed TEM gene, and only 12 (14.1%) possessed the SHV gene (Table 7). Furthermore, CTX-M and TEM genes together were presented in 15.3% of the ESBL isolates while CTX-M together with SHV genes were presented in 8.2% and SHV combined with TEM were found in 3.5% of the total ESBLs.

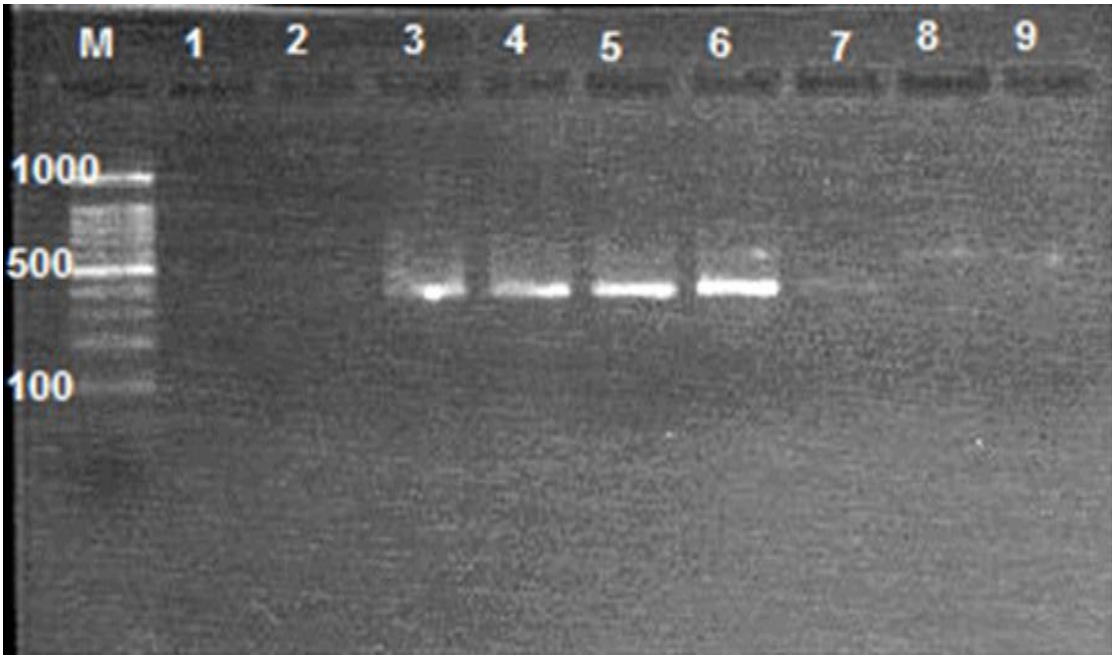

Figure 11. 2% Agarose gel electrophoresis of PCR bla SHV genes products. Lane M: 100 bp molecular weight marker, Lane 1: negative control, Lane 3: *bla* SHV positive control *K. pneumoniae* KPN05 (392 bp), Lanes: 2, 7, 8, 9 are tested isolates showing negative results for *bla* SHV gene, Lanes 3, 4, 5 and 6 are tested isolates with positively amplified *bla* SHV gene.

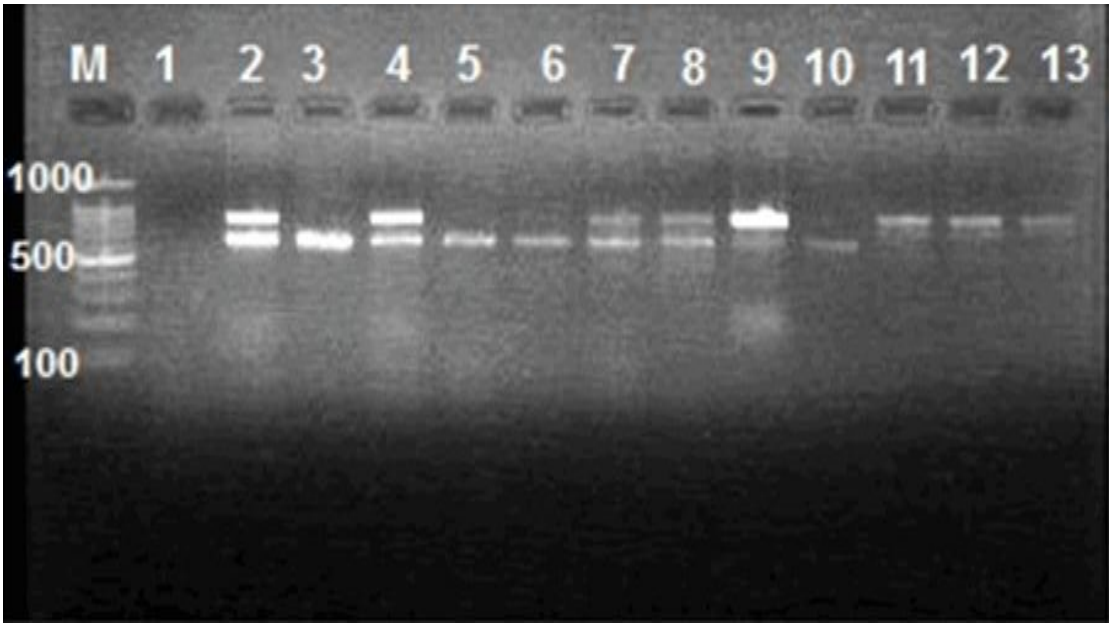

Figure 12. 2% Agarose gel electrophoresis of PCR bla CTX-M and bla TEM genes products. Lane M: 100 bp molecular weight marker, Lane 1: negative control, Lane 2: TEM positive control (512 bp) and CTX-M positive control (619 bp), Lanes 3, 5, 6 and 10 tested isolates showing positive results for TEM gene, Lanes 9, 11, 12 and 13 are tested isolates with positively amplified *CTX-M gene*, Lane 4, 7 and 8 tested isolates showing positive results for both CTX-M and TEM genes

Table 7. Frequency of ESBL genes among 85 target isolates.

Gene	*E.coli*(66)	*K.pnemoniae*(19)	Total (85)
CTX-M	57 (86.4%)	6 (31.6%)	63 (74.1%)
TEM	19 (28.8%)	8 (42.1%)	27 (31.8%)
SHV	3 (4.5%)	9 (47.4%)	12 (14.1%)
CTX-M +SHV	2 (3.0%)	5 (26.3%)	7 (8.2%)
CTX-M + TEM	10 (15.2%)	3 (15.8%)	13 (15.3%)
SHV + TEM	1 (1.5%)	3 (15.8%)	4 (4.7%)

CHAPTER FIVE

DISCUSSION

Extended-spectrum beta-lactamase (ESBL) production is an increasing antibiotic resistance problem. Moreover, the production of ESBL by Enterobacteriaceae is an increasing challenge in the treatment of infections. In Saudi hospitals, the rate of resistance to beta lactamase grew rapidly in the most of the admitted patients (Kadar et al., 2005). In our study in relation to demographic and personal data, according to gender the females (75%) were found to be significantly more than the males (25 %) much findings have been previously reported by many authors worldwide. In Pakestan, regarding the gender distribution ESBLs -producing isolates were more common in females,106 out of 165 (64.3%) as compared to males,59 out of 165 (35.7%)(Shamim *etal*,. 2006). Also,another study in Pakistan regarding gender classification in case of β-lactamase-producing *E.coli*, females (60%) had a higher incidence as compared to males (40%). Similarly, in case of *Klebsiella*, females (55%) and males (45%) were effected(Saba *etal*., 2011); in Italy(Milan) nearly 80% of all isolates were from women (female to male ratio =3:8)and 58% from subjects aged 60 years or more (Magliano *etal*., 2012);in Abia State Nigeria,ESBLs were found to be more prevalent in females than males (Nwosu*etal*., 2014);in Nepal in term of Gender distribution 443 (25.6 %) were male and 1287 (74.4%) were female hence the ratio is 0.34:1, respectively and in another study also in Nepal has been found that in study enrolled 321 suspected UTI patients where 62.92% suffered from UTIs in which 48.01% were male and 51.98% female (Khushbu Yadavand Satyam Prakash, 2017).Different studies revealed a significant increased in the female preponderance for ESBL production among the study subjects (Kiratisin *et al*., 2008 and Metri *e tal*., 2011). This may attributed to the fact that the females possessed high

tendency to acquire diseases such as UTIs with high frequency comparing to the males. Whilst the high ratio of ESBLS were obtained from patients with UTIs in this. In our study results reflected significantly high rate of resistance among ESBL isolates compared with non-ESBL producers ($P < 0.05$) and the antibiotic sensitivity pattern of Gram negative bacteria (Table 5) revealed that the maximum resistance was seen against Cephalothin, Ampicillin, Aztereonam with a percentage of (95.3%) respectively, followed by Ciprofoxacin 62 (72.9) and Trimethoprime-Sulfamethaxazole 61 (71.8). These results were found to be in accordance with many researchers worldwide (Keah et al., 2007 ; Akram et al., 2007 and Manjunath et al., 2011).

In the present study, a total of 359 Gram negative Enterobacteriaceae strains were isolated from various clinical specimens among which Extended spectrum β-lactamases producers (ESBLs) were isolated with high prevalence 85 out of 359(23.7%) (Figure 9). ESBLs were found to be more frequent in urine specimens (61.7%), followed by blood (15.1%), pus (8.2%), wound (5.5%), sputum (4.1%), eye (2.7%) and high vaginal swap (2.7%)(Figure 10).These findings were previously stated in the Eastern Province of Saudi Arabia by Kader et al., (2005). These authors recorded almost the same ratio of ESBs obtained from patients with different clinical manifestations. The high frequency of ESBLs among urine specimens may be attributed to the high prevalence of UTIs comparing to the other infections. Additionally, this may also attributed to the shortage of policies and regulations towards antibiotics intake in this part of Saudi Arabia.

Also our study recorded high ratio of ESBLs isolated from Intensive Care Unit (30.6%), followed by Urology unit (24.7%), the medium frequency was reported in family medicine and surgery department (11.7%). While the lowest was in Obstetrics and gynecology ,Pediatric,

Respiratory, Internal medicine, Heamodialysis,Neonate with percentages less than 3%(Table 6).Data obtained from this project seemed to be running with style totally agreed with those mentioned by Kader and co-authors(2005). In our project infection has relation to age groups especially those who are in late life age groups,very young age groups or in reproductive age groups 20-39 years because the patients become immune-compromised and there are greater opportunities that to suffer from infection epecially females has more chances to be infected with UTI and also nosocomial infections caused by ESBL producing pathogens are associated with risk factors such as elderly age, prolonged hospitalization, previous antibiotic use, and presence of invasive devices (Bradford,2001; Panhotra,*etal*,2004). Although in this project The demographics study indicates that old age (> 60 years) of age groups 32(37.6%) (Table 3) consider the highest frequency, this was similar to that study in Eastern Province Saudi Arabia .The majority of the ESBL isolates (229/409; 56%) were identified in patients older than 60 years, followed by 31.5% (129/409) in those aged 21 to 60 years, 7.1% (29/409) from the 11- to 20-year-old group, and 5.4% (22/409) in those younger than 10 years. It is significant that, although 56% of ESBL isolates were identified in patients older than 60years, this age group constituted of only 16.2% (1,094/6,750) of the total number of patients from whom *E. coli* and *Klebsiella spp* were isolated during the study period (Kader etal,2005).In Pakestan In 165 ESBLs-producing isolates, the age of the patients was known which varied from 3 months to 70 years. ESBLs-producing Enteric gram negative rods were most frequent in 61–70 years of age group, 46 out of 165 (27.9%), 41–50 years of age group, 33 out of 165 (20.0%) , followed by 51–60 years of age group, 28 out of 165 (16.9%)(Shamim *etal*,2006).

This study clearly explained that previously expoure to antibiotics treatment against positive ESBL participants, (71) 83.5% were more than in those with no exposure to antibiotics treatment (14) 16.5% ,also the frequency of female (51) 60% more than male (34) 40% (Figure6).InUSA there is asimilar result indicated that After controlling for potential confounders, the relative risk of having an extended-spectrum-beta-lactamase-producing isolate rather than a susceptible isolate was 2.2 times higher among those with antibiotic exposure in the 30 days prior to infection than in those with no antibiotic exposure(Danielle M Zerr etal,2016). Also in Greece there was study showed that the risk of infection with ESBL-CRKP rose with increasing duration of prior treatment with β-lactam/β-lactamase inhibitor combinations, fluoroquinolones and carbapenems(Evangelos etal,2011).

Although research regarding ESBL producers drew attention regionally and globally, data in this regard in Al-Madenah Al-Monawwarah area is still limited. Thus, we aimed to shed light on the ambiguities surrounding this important Arabian Gulf region, high ESBL prevalence of 31.7% in Kuwait and 41% in the United Arab Emirates(Mokaddas *et al.,2008;* Al-Zarouni *et al.*,2008) has been reported among inpatients. For Saudi Arabia, reported ESBL rates vary from 8.5-38.5% (El-Khizzi and Bakheshwain,2006; Babay,2002).Thus, in comparison to regional data, the finding of 85/359 (23.7%) ESBL producers in this study are on the middle of the spectrum. This finding is also similar to data reported from surveys in other countries in Europe and Asia(Ishikawa *et al.,2003;*Ishikawa *et al*.,2003).

Although in our study many species of Enterobacteriacae were isolated from different samples, ESBLs were identified only among *E. coli* (66/85; 77.6%), and *K. pneumoniae* (19/85; 22.4%). In this study, the predominance of *E. coli* is similar to, but the frequency is less than what

has been reported in data from the Eastern Province of Saudi Arabia (83%) (Khanfar,2009).Other results obtained from different parts of the world demonstrated that *K. pneumoniae* is the most frequent pathogen compared to *E. coli* (52.27%: 46.43%) (Kaur and Aggarwal,2013). Moreover, we reported in this research that ESBL- producers are significantly resistant to other drugs that do not belong to beta lactam antibiotics ($P < 0.05$). Previous studies have reported the occurrence of this phenomenon from other parts of the world like India(Kaur and Aggarwal,2013) and Sudan (Omar *et al*,.2013).

Our data confirmed the existence of the *bla*CTX-M gene in Al-Madenah Al-Monawwarah with a significant ratio (74.1%)(Table7). Similar findings were reported from Riyadh, KSA (Babay,2002 ; Al-Agamy *et al*,.2009) and Makkah (Alyamani,2015). High prevalence of *bla*CTX-M were also reported from other parts of the world; 71.4% in Sudan, 83.3% in Spain, 41.3% in Mongolia, 59.0% in Bangladesh, 48.5% in India, 50.0% in Taiwan, 84.0% in Thailand and 35.9% in Russia (Omar *et al*,.2013; Colom ,.*et al* ; Edelstein ,. *et al*,.2003) . Our findings proved for the first time the high occurrence of CTX-M gene among different ESBL-producing isolates in this special and overcrowded part of Saudi Arabia. Other ESBL genes were also found among the isolates from the enrolled subjects, the results were as follows: SHV gene among all ESBL producers was (4.5% in *E. coli* and 47.4% in *K. pneumonia*) and TEM gene (28.8 % in *E. coli*, 42.1% in *K. pneumoniae*), while 15.3% of both *E. coli* and *K. pneumoniae* present as co-producers of CTX-M with TEM, 8.2% and 4.7% as co producers of CTX-M with SHV and SHV with TEM ESBL genes respectively (Table7). Many authors in different part of the world have proved similar results (Bradford,2001;Lewis *et al*,.1999).

From this research we can conclude that there is a relatively high proportion of ESBL producers in Al-Madenah Al-Monawwarah (23.7%) compared to other parts of KSA, which will be considered as a major challenge to the health authorities in this region. Putting into account the high emergence of CTX-M gene (74.1%) among the ESBLs of the study subjects, which is known as a serious public health concern worldwide and has been noted to be the cause of outbreaks as reported elsewhere.

All the 359 Enterobacteriaceae isolates that identified by BD Phoenix 100 and showed ESBL positive were subjected to the screening disc diffusion test,and followed by confirmatory disc diffusion testing.

Chapter SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

Considering various findings of the present study, it can be concluded that:

6.1.1. There is a relatively high proportion of ESBL produceing-Enterobacteriacae in Al-Madenah Al-Monawwarah (23.7%) compared to other parts of KSA, which will be considered as a major challenge to the health authorities in this region.

6.1.2. The high emergence of CTX-M gene among included ESBLs (74.1%).

6.1.3. Based on the genetic basis in this study, it is possible to conclude that the distribution of β-lactamase genes in Almadinah Almonawarah is very similar to the gene variants found elsewhere in the world.

6.1.4. The prevalence rate of ESBLs β-lactamase and resistance to multiple antibiotics were noticeable among most of isolates,especially *E.coli* and *K.pnemoniae.*

6.1.5. The ESBL-positive strains some carried only one genes (CTX-M,TEM or SHV) and some more than oneCTX-M +SHV,CTX-M+TEM and SHV+TEM genes.

6.2. Recommendations

The study recommended the following:

6.2.1 The early detection and reporting of suitable antibiotics can reduce the treatment failure in ESBL-producing bacteria.

6.2.2. Continuous monitoring system in hospitals, community and effective infection control measures to prevent the rapid spread.

6.2.3. Rational use of antimicrobial or ideal antimicrobial use of correct drug by the best route in right dose at optimum intervals for the appropriate period and after an accurate diagnosis.

6.2.4. Molecular detection and identification of β- lactamases would be essential for reliable epidemiological investigation of antimicrobial resistance.

6.2.5. Surveillance with large and variable clinical samples from different parts of the country for mapping ESBLs in KSA.

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Appendix I

questionnaire

Shendi University

College of Graduate Studies and Scientific Research

Phenotypic Detection and Molecular Characterization of Extended-Spectrum β-lactamases Producing Enterobacteriaceae in Al-Madinah Al-Monawwara Region, KSA

Questionnaire for Requirement of PhD Degree

1.0patient details

1.1Age _____

1.2Gender M ☐ F ☐

1.3ID number _____

2.0Details of hospitalization

2.1Name of hospital _____

2.2Date of admission ____/_____/_____

2.3Patient ‘s primary diagnosis _____

2.4Word /unit name _____

3.0Details of infection

3.1 what type of infection ? UTI ☐other☐

If other _____

3.2 why was the culture done ?

3.3 is it the first time ?

3.4 did the patient has drug ? use of antibiotic in the past six months?

antibiotic given for the first episode:

Antibiotic given date started Duration of treat Route of admission

…… ……. ……. ……..

Hospital admission in past six months yes ☐ No ☐

3.5 please provide details of antibiotic treatment given now :

Culture date : _____/_____/____ reason for culture _____

Type of antibiotic …….

Sensitive antibiotics :

Resistant antibiotics :

Appendix II

Preparation of Media

Brain Heart Infusion (BHI) broth

Composition	g/l
Calf Brain infusion solid……	12.5
Beef Heart infusion solid……	5.0
Glucose……	2.0
NaCl ……	5.0
Di-sodium phosphate……	2.5

Preparation

Thirty-seven gm of dehydrated Brain Heart Infusion agar medium (Hi-media, Indiawas suspended in 1000 ml distilled water and dissolved the medium completely. Thesolution was then sterilized by autoclaving, for best result, the mediumshould be usedon the day it is prepared, otherwise, it should be boiled or steamed for a few minutesand then cooled before use.

To prepare preserved media with 20 % of glycerol

Add 20 ml of glycerol to 80 ml of Brain heart infusionbroth media. Distribute 1.5 ml into autoclavable eppendroff tubes. Sterilize by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes.

Blood agar medium

Composition

Ingredients	**gram/liter**
Heartinfusion……	500.00
Tryptose……	10.00
Sodium chloride……	5.00
Agar……	15.00

Preparation

Forty grams of the dehydrated blood agar medium was suspended in 1000 ml colddistilled water in a flask and boiled to dissolve the medium completely. It was thensterilized by autoclaving at 1210C and 15 lbs pressure for 15 minutes. The autoclavedmaterials were allowed to cool to a temperature of 450C in a water bath. Defibrinated5-10% sheep blood was then added to the medium aseptically and distributed tosterile petridishes. Sterile media was stored in refrigerator at 40C for future use.

Muller Hinton agar medium (pH 7.4)

Composition

Ingredients	**Gram/liter**
Beef dehytrated infusion	300
Casein hydrolysate	17.50
Starch agar	17.00
Agar	17.00

Preparation

Thirty-eight grams of dehydrated Mueller Hinton agar medium was suspended in1000 ml cold distilled water and boiled to dissolve the medium completely. Thesolution was then sterilized by autoclaving at 1210C and 15 lbs pressure for 15minutes. The autoclaved media was stored at 40C

MacConkey agar

2.1 Formula of MacConkey's Agar (pH 7.1)

Ingredients	**gram/liter**
Bacto peptone	17.0 g
Proteose peptone	3.0 g
Lactose	10.0 g
Bile salts mixture	1.5 g
Sodium chloride	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Distilled water	1,000.0 ml

2.2 Preparation

Media was prepared according to instruction of manufacture as follow. 55.0 grams were suspended in 1000ml distilled water. The preparation heated to boiling to dissolve the medium completely, then sterilized by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes. After cooling to 50-55, and 20-25 mL of molten preparation were poured into sterile

disposable 90 mm in diameter Petri dishes, (Makie and MacCarteny, 1996).

CLED Agar (Cysteine Lactose Electrolyte Deficient)

Formula in grams per liter (PH 7.4)

Lactose.....10,00

Gelatin Peptone 4,00

L-Cysteine 0,128

Bacteriological Agar 15,00

Casein Peptone 4,00

Beef Extract..... 3, 00

Bromothymol Blue 0,02

Preparation

Suspend 36 grams of the medium in one liter of distilled water. Soak 10-15 minutes and mix well. Heat slowly while stirring frequently boils for a minute. Sterilize in the autoclave at 121°C (15 lbs. of sp.) for 15 minutes. Pour into Petri dishes. When the medium is solidified, invert the plates to avoid excess moisture.

XLD agar Ph 7.4

Ingredients	**gram/liter**
Yeast extract……....	3.0
L-Lysine HCl……	5.0
Xylos…….	3.75
Lactose……..	7.5
Sucrose……..……	7.5
Sodium desoxycholat….……...	1.0
Sodium chloride……..……..	5.0
Sodium thiosulphate……..	6.8
Ferric ammonium citrate……...……	0.8
Phenol red……..	0.08
Agar…….	12.5

2.2 Preparation

According to instruction of manufacture 53g was Suspend in 1 litre of distilled water. It was Heated with frequent agitation until the medium boils. Do not over heat.And transfered immediately to a water bath at 50°C.Then poured into sterile Petri dishes as soon as the medium has cooled

Nutrient agar

Formula in grams per liter

Peptone ……...1.0 g
Sodium chloride…….……...5 g
Beef extract …….…….….3 g
Agar ……..20 g
Distilled water ……1000 ml
pH ……6.8

Preparation:

Dissolve all components in distilled water except agar. Adjust the pH. Add the agar. Sterilize in autoclave at 121°C for 20 min.

Kligler Iron Agar

Formula in grams per liter

Peptone mixture 20,00

Sodium Chloride..... 5,00

Ferric Ammonium Citrate 0,50

Phenol Red ..... ,0,025

Lactose 10,00

Dextrose 1,00

Bacteriological Agar 15,00

Sodium Thiosulfate.....0,50

Preparation

Suspend 52 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation. Boil for one minute. Dispense into tubes and sterilize at 121º C (15lbs. pressure) for 15 minutes. Allow to cool in a slanted position so as to obtain butts of 1’5-2 cm. Depth. For greater

accuracy, Kligler Iron Agar should be used on the day of preparation or melted and solidified before use.

Motility test

Requirements

Semisolid agar

Method

1. Astraight needle was taught to acolony of a young (18- to 24- hour) culture growing on agar medium.

2. Stab once to a depth of only 1/3 to 1/2 inch in the middle of the tubewas stapped and incubated at 35° to 37° C ,

Results

Positive: Motile organisms was spreaded out into the medium from the site of Inoculation.Negative: Nonmotile organisms remain at the site of inoculation.

Quality Control

Positive: *Escherichia coli*

Negative: *Klebsiella pneumonia*

Simmons Citrate Agar

Formula in grams per liter (PH 7)

Ammonium Dihydrogen Phosphate 1,00

Dipotassium Phosphate..... 1,00

Sodium Chloride.....5,00

Sodium Citrate 2,00

Magnesium Sulfate.....0,20

Bacteriological Agar..... 15,00

Bromothymol Blue.....0,08

Preparation

Suspend 24,3 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation until completely dissolved. Dispense

in tubes and sterilize in the autoclave at 121ºC (15 lbs sp.) for 15 minutes. Cool the tubes in a slanted position so that the base is short (1-1,5 cm. deep). Alternatively, the media can be poured into petri plates.

Christensen's Urea Agar

Formula in grams per liter (PH 6.9)

Gelatin Peptone..... 1,00

Dextrose 1,00

Sodium Chloride.....5,00

Monopotassium Phosphate 2,00

Urea 20,00

Phenol Red.....0,012

Preparation

Dissolve 29 grams of the medium in 100 ml. of distilled water. Sterilize by filtration. Separately dissolve 15 grams of agar in 900 ml. of distilled water by boiling. Sterilize in autoclave at 121°C (15 lbs.sp) for 15 minutes. Cool to 50°C and add to the 100 ml. of the sterile Urea Agar Base. Mix well and dispense aseptically in sterile tubes. Leave the medium to set in a slanted position so as to obtain deep butts. At a pH of 6.8 to 7.0 the solidified medium should have a light pinkish yellow color. Do not remelt the slanted agar.

Kligler Iron Agar

Formula in grams per liter

Peptone mixture 20,00

Sodium Chloride..... 5,00

Ferric Ammonium Citrate 0,50

Phenol Red 0,025

Lactose 10,00

Dextrose 1,00

Bacteriological Agar 15,00

Sodium Thiosulfate.....0,50

Preparation

Suspend 52 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation. Boil for one minute. Dispense into tubes and sterilize at 121º C (15lbs. pressure) for 15 minutes. Allow to cool in a slanted position so as to obtain butts of 1’5-2 cm. Depth. For greater accuracy, Kligler Iron Agar should be used on the day of preparation or melted and solidified before use.

Appendix III

Reagents and Stains

Gram Stain (Cheesebrough, 2007)

Most bacteria can be differentiated by their Gram reaction due to differences in the cell wall structure into Gram positive which after being stained dark purple with crystal violet are not decolorized by acetone or ethanol and Gram negative which after being stained with crystal violet lose their color when treated with acetone or ethanol and stain red with Saffranin.

Requirements

Crystal violet Gram stain (Hi Media)

To make 1 liter:

Crystal violet……..... ... . …20 g

Ammonium oxalate…… .....……9 g

Ethanol or methanol, absolute…… .....… ...….95 g

Distilled water…… to 1 liter

Lugol's iodine (Hi Media)

To make 1 liter:

Potassium iodide…….....……20 g

Iodine…….……..10 g

Distilled water…….…….. To 10 liter

70% alcohol

Absolute alcohol…….....…..70 ml

Distilled water…….....…….30 ml

Saffranin (HiMedia)

Method of Preparation

• The dried smear was fixed by heat.

• The fixed smear was covered with crystal violet for 30-60 minutes.

• The stain was washed off with clean water.

• All water was tipped and the smear covered with lugol's iodine for 30-60 minutes.

• The stain was washed off with clean water.

• 70% alcohol was rapidly applied for 10-20 seconds for decolourization and then washed rapidly with clean water.

• The smear then covered with Saffranin stain for 2 minutes.

• The stain was washed off with clean water, back of slide was cleaned.

• After air-dry, smear was examined microscopically by using X 100 lens.

Results

All Enterobacteriaceae appeared as gram negative rod.

Preparation of Turbidity Standard

1- 1 % v/v solution of sulpharic acid was prepared by adding 1 ml of concentrated sulpharic acid to 99 ml of water. Mix well

2- 1.17% w/v solution of barium chloride was prepared by dissolving of 2.35g of dehydrate barium chloride ($Bacl_2.2H_2O$) in 200ml of distilled water.

3- To make the turbidity standard 0.5 ml of barium chloride solution was added to 99.4 ml of the sulpharic acid solution. Mix well

4- A small volume of the turbid solution was transferred to screw-caped bottle of the some type as used for preparing the test and control inoculate (Mackie and McCartney, 1996).

Oxidase test reagent

Dimethyl-p-phenylene diamine hydrochloride …….1.0 g

Distilled water ……100.0 ml

The reagent should be made fresh daily. It should not be stored longer than one week in the refrigerator.

If the preparation becomes darkened, discard. Tetramethyl-p phelylenediamine dihydrochloride (1%) is even more sensitive but it is more expensive and difficult to obtain.

Indole productionTest

Requirements

Tryptophan Culture Broth

Formula in grams per liter (PH 7.5)

Casein Peptone 10,00

L-Tryptophan 1,00

Sodium chloride.....5,00

Preparation

Suspend 16,0 grams of medium in one liter of distilled water. Heat to boiling agitating frequently. Distribute in test tubes, 3 ml each. Close the tubes with cotton or with a plastic or metallic cap. Sterilize at 121º C (15 lbs. sp.) for15 minutes.

Preperation of kovac'Reagent

Kovac's reagent was prepared by dissolving 10 gm of p-dimethyl aminobenzaldehyde in 150 ml of isoamyl alcohol and then slowly adding 50 ml of concentrated hydrochloric acid

Appendix IV

PCR Reagents

Gel electrophoresis reagents

Buffers preparation:

10X TBE buffer:

Tris base	108g	0.89M
Boric acid	55g	0.89M
0.5M EDTA	4.6g	20Mm

pH 8.3

dH_2O 1000ml

Preparation 1.5% agarose gel preparation for genomic DNA

0.75g was completely dissolved in 50 ml 1X running buffer by heating, the melted gel then was allowed to cooled to 50 C^o, where 3 drops of ethidium bromide(10mg\ml) was added to melted agarose

Ethidium bromide

Ethidium bromide was prepared by dissolving 1g of ethidium bromide in 100ml distilled water and was kept in brown bottle.

Loading dye: Was prepared by mixing

30% v/v glycerol,

25% w/v bromophenol blue

25% w/v xylene cynol.

Store in $4C^0$ to avoid mold growing in sucrose.

Gel electrophoresis procedure

The melted agarose containing ethidium bromide was poured into gel electrophoresis running tank and was allowed to solidify. The comb was removed and 1X running buffer was added. 2µl of loading dye was mixed with 1µl of DNA marker. The mixture was then placed in the gel well. To each of others gel wells a mixture containing equal volume of loading dye

and amplified genomic DNA was applied. The electrophoresis tank was connected to power supply and switch on and the voltage was adjusted to 75V for 30 minutes. Following gel running the bands of DNA was visualized by using ultraviolet light in gel documentation system.

PCR reaction buffer (-10X standard reaction buffer (100 mM Tri-HCl pH8.5, 500Mm KCl, 15 Mm $MgCl_{2,}$1% Triton X-100)

-$MgCl_2$ 25mM

dNTPase Nucleotides(mixed) (100mM 0.8 ml)

Tag DNA polymerase

DNA (marker) ladder size rang (100—1,000 bp) Solis BioDyne, GERMANY

Appendix V

PCR Machines

PCR equipments

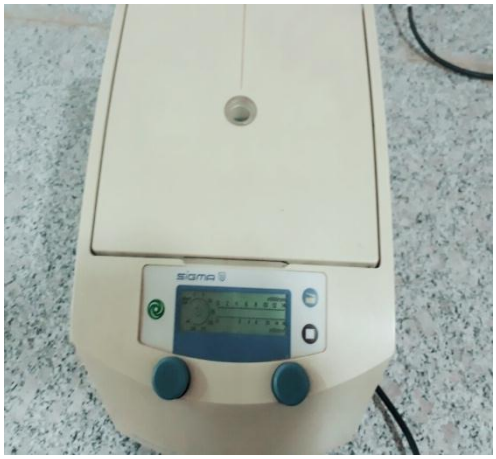
Power supply blue power 500 SERVA.

Sigma centrifuge 1---15 GERMANY.

Water bath scott science UK.

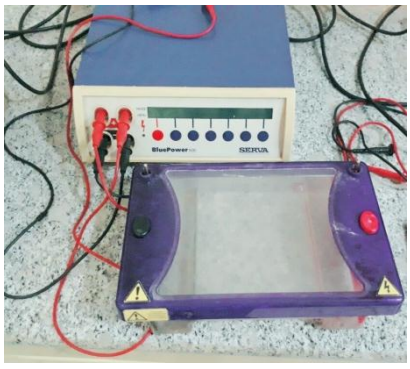
PCR machine technique.

Synger gel decommentation system, synoptiesl LTD, UK.

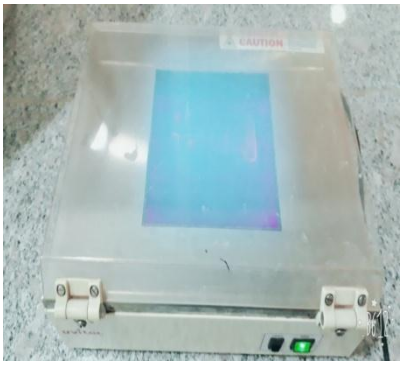


Microcentrifuge Device

Thermocycle Device

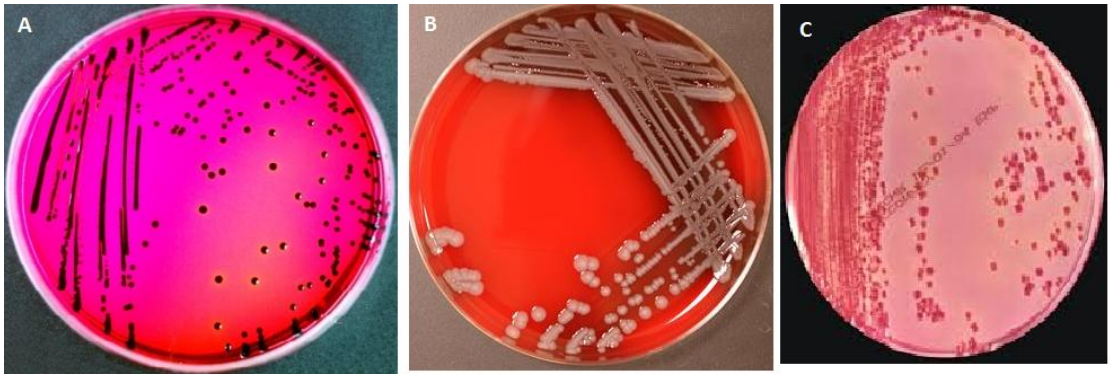


Gel Electrophoresis and Power Supply Device

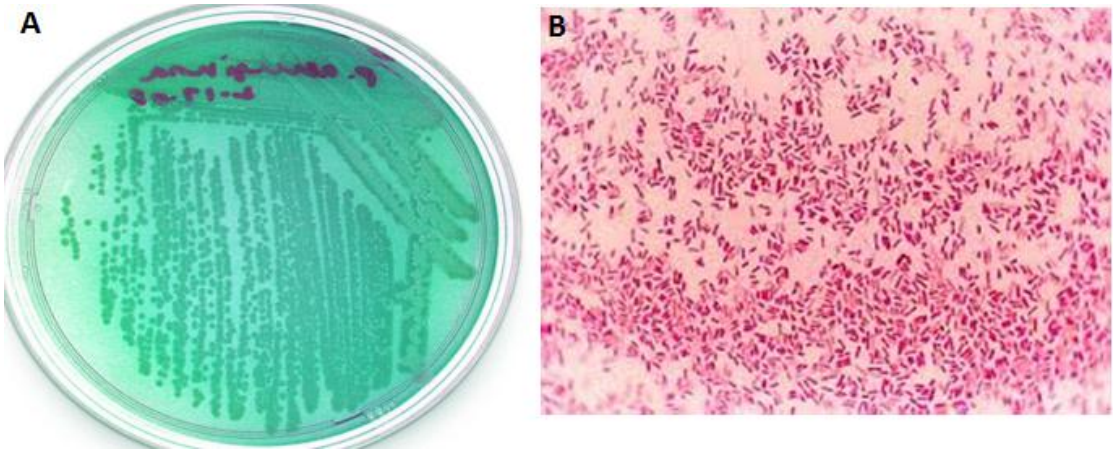


UV Light Transilluminater Device

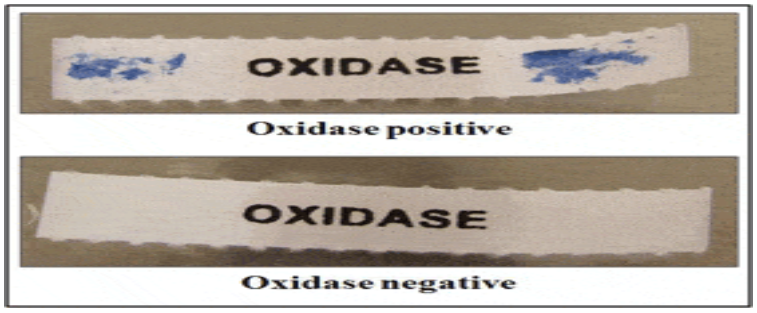
Appendix VI
Colour plates



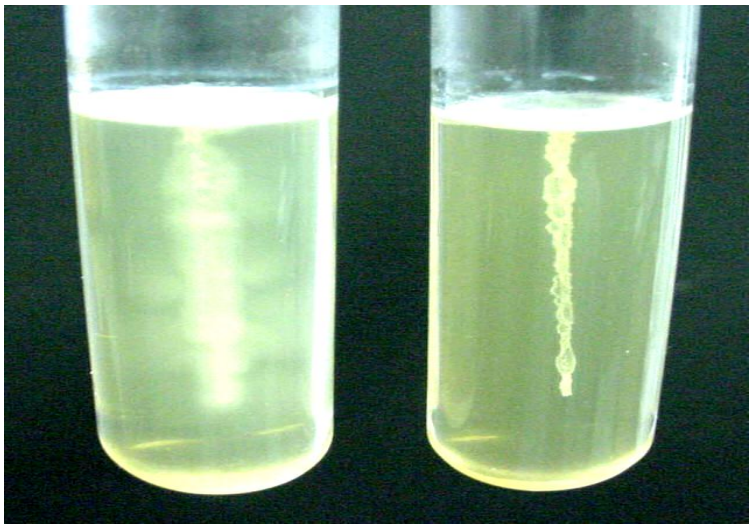
Colour plate 1. A 24 hr old culture of the isolated bacteria on: A) XLD medium showing pink colonies with black center; B) on blood agar showing large non-hemolytic colonies; C) on MacConkey agar showing pink lactose fermenter colonies.



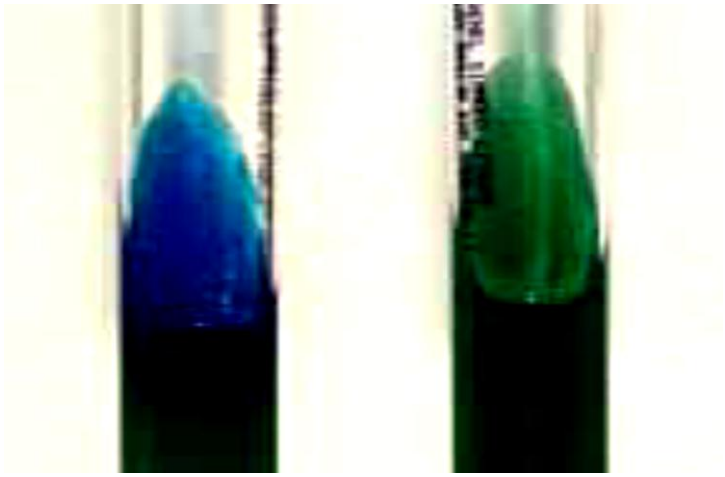
Colour plate 2. A: 24 hr old culture of isolated bacteria on CLED medium showing green lactose non fermenter colonies; B: Gram's stain showing gram –ve rods of isolated bacteria obtained from enrolled subjects.



Colour plate 3. Oxidase test. Positive (Blue colour) .Negative (No clour change).All *Enterobactericae* isolated from enrolled patients they were showed Oxidase negative.



Colour plate 4. Motility test. Left motile showed by *E.coli,salmonella species,Prot species,Citrobacter,Enterobacter,Serratia,Providenciaspecies and Yercinia enterocolytica.eus*



Colour plate 5. Citrate utilization test.Showed positive (Blue clour) by *Klebsiella pneumonia , salmonella species Serratia* and *Providenciaspecies*.

Colour plate 6. Urea hydrolysis (Christensen's method).Left:Positive it was showed by P*roteus species,Morganella morganii, Klebsiella pneumonia and Yersinia enterocolytica*,Right:Negative.

Colour plate 7. Indole test. Right,Positive.Showed by *E.coli,Protrus vulgaris,Morganella morganii and providencia species.*

A B C D

Colour plate 8. Kligler Ion Agar.It was used for identification of *enterobacteriacae*from the left the tube showed **A,** Acid slant/acid butt with gas, no H2S (A/A) it was *E.coli*.**B,** Alkaline slant/acid butt, no gas, (K/A) It was *Yercinia enterocolytica*.**C,** Alkaline slant/acid butt no gas, H2S-positive (K/A + H2S) produced by *Proteus vulgaris* and *Salmonella typhi D*,Red/slant/Red butt(Red/Red) it was control negative.