بسم الله الرحمن الرحيم


Sudan University of Science and Technology

College of Graduate Studies


# Detection of Extended Spectrum Beta- Lactamase among Gram-negative Bacteria Isolated from Patients in Omdurman Military Hospital

**الكشف عن انزيم البيتاالاكتاميز في البكتريا سالبة الجرام المعزولة من المرضي في مستشفى أم درمان العسكري**

A Dissertation submitted for partial fulfillment of the requirements of M.Sc. Medical Laboratory Science (Microbiology)


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# الآيــــــــــــة

**قال تعالي:**

(فَلَمَّا اسْتَيْأَسُواْ مِنْهُ خَلَصُواْ نَجِيًّا قَالَ كَبِيرُهُمْ أَلَمْ تَعْلَمُواْ أَنَّ أَبَاكُمْ قَدْ أَخَذَ عَلَيْكُم مَّوْثِقًا مِّنَ اللَّهِ وَمِن قَبْلُ مَا فَرَّطتُمْ فِي يُوسُفَ فَلَنْ أَبْرَحَ الأَرْضَ حَتَّىَ يَأْذَنَ لِي أَبِي أَوْ يَحْكُمَ اللَّهُ لِي وَهُوَ خَيْرُ الْحَاكِمِينَ)

صدق الله العظيم

سورة يوسف الآية (80)

# DEDICATION

To my father, mother, brothers and sisters

## ACKNOWLEDGEMENT

The completion of this research could not have been possible without the participation and assistance of so many people whose names may not all be enumerated. Their contributions are sincerely appreciated and gratefully acknowledged.

I would like to express may deep appreciation and indebtedness to supervisor: Prof. Humodi Ahmed Saeed for endless support, advice, patience, and continues follow-up.

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To all relatives, friends, and who in one way or other shared their support, morally, financially and physically, thank you.

## LIST OF ABBREVIATIONS

AES: Advanced Expert System

AmpC: Ampicillinase C

ATCC: American Type Culture Collection

AST: Antimicrobial susceptibility test

bla: β-lactamase Coding gene

CLSI: Clinical Laboratory Standard Institute

CTX-M: Cefotaximase, Munich

DNA: Deoxyribonucleic Acid

ESBL: Extending Spectrum Beta-lactamase

GES-2: Intgron-brone gene from *Pseudomonas*

Int: International

KPC-1: Carbapenem Resistant Strain of *Klebsiella pneumoniae*

MICs: Minimal Inhibitory Concentrations

NTPs: Deoxynucloside Triphosphate

ODM: Omdurman Military Hospital

PCR: Polymerize Chain Reaction

Rev: Review

SHV: Sulfhydril Variable

SPSS: Statistical Package for Social Science

TEM: Temoniera

ABSTRACT

The antibiotics resistance is worldwide problems recently especially in developing countries. The aim of the present study was to detect Extended- spectrum beta-lactamase (ESBL) among bacteria isolated from different clinical specimens. This study was carried out during period from April to August 2018.

A total of 70 isolates were collected from Omdurman Military Hospital previously isolated from urine 65(65%), wound swab 20(20%), and sputum 15(15%). The isolates were isolated from both males 62(62%) and females 38(38%). The age of participants were ranged from 2 to 89 year. The isolates were reidentified by their colonal morphology, Gram stain, and biochemical tests. Identified bacteria were subjected to antimicrobial sensitivity tests. The antibiotics used were: ceftrixone, cefotaxime, cefepime, ceftazidime, and azteronam. The presence of ESBLs were detected by double disc synergy test and combined disk test.

The results revealed the identified bacterial isolates were *Klebsiella pneumoniae* 20(28.5%) *Escherichia coli* 40(57.1%) *Pseudomonas aeruginosa* 8 (11.4%), and *Proteus* species 2 (2.8%). ESBLs were detected in 15(21.4%) bacterial isolates. These were 14(20%) *E.coli* and 1(1.4%) *K. pneumoniae*. The positive ESBLs producers bacterial isolate were obtained from male 9(12.8%) and female 6(8.6%).

The frequency of ESBLs producers among the age group were 5(7.1%) in youth, 7(10%) in adult, and 3(4.3%) in elderly.

The study concluded that there is high ratio of ESBLs among isolated bacteria in Omdurman Military Hospital. The most ESBLs producers are *E. coli*. Further studies are required using large number of isolates and advanced techniques to validate the results of this study.

## المستخلص

مقاومة المضادات الحيوية هي مشاكل عالمية هذه الأيام، خاصة في البلدان النامية . الهدف من هذه الدراسة هو الكشف ان انزيمات البيتالاكتام واسعة الطيف (ESBL) بين البكتيريا المعزولة من العينات السريرية المختلفة .أجريت هذه الدراسة خلال الفترة من أبريل إلى أغسطس .2018 تم جمع 70 عزلة من مستشفى أم درمان العسكريعزلت سابقا وهي بول 65 (65%) ومسحة جرح(20%) 20 وقشع %) 15 (15. تماعادة التعرف على العزلات من خلال اختبارات تشكيل القولون ، صبغة جرام ، والاختبارات البيوكيميائية .تم إخضاع البكتيريا التي تم تحديدها لاختبارات الحساسية للمضادات الميكروبية .المضادات الحيوية المستخدمة هي : سيفتريكسون ، سيفوتاكسيم ، سيفيبيم ، سيفتازيديم ، وأزيترونام .تم الكشف عن وجود منتجي ESBLs عن طريق اختبار تآزر القرص المزدوج واختبار القرص المشترك .أظهرت النتائج أن 70(70% )من العينات أعطت نمو جرثومي ، بينما 30 (30%) (لم تظهر أي نمو بكتيري .تم عزل 38(54.2%) من البكتيريا من البول 14(20%) من مسحات الجروح و 18(25.7%) من البلغم .كانت العزلات البكتيرية التي تم تحديدها هي (28.5% 20 *Klebsiella pneumoniae* *Escherichia coli* 40 (57.1%)) *pseudomonas aeruginosa* 8(11.4%) ) *Proteus* 2 (2.8%).

تم الكشف عن ESBLs في21.4) 15 ٪ (عزلة بكتيرية .كانت هذه 14 20)٪(بكتريا قولونية و1.4) 1 ٪ (بكتريا رئوية .تم الحصول على منتجي ESBLs الموجبة12.8) 9 ٪ (من الذكور و8.6) 6 ٪ (من الإناث .كان تواتر منتجي ESBL بين الفئة العمرية 7.1) 5 ٪ (في الشباب ، و 10) 7 ٪ (في البالغين ، و 4.3) 3 ٪(في كبار السن) .

وخلصت الدراسة إلى أن هناك نسبة عالية من الانزيمات البيتالاكتام بين البكتيريا المعزولة في مستشفى أم درمان العسكري .معظم منتجي ESBLs هم *E.coli*. تتطلب المزيد من الدراسات استخدام عدد كبير من العينات والتقنيات المتقدمة للتحقق من صحة نتيجة هذه الدراسة.

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# CHAPTER ONE

# INTRODUCTION AND OBJECTIVES

# CHAPTER ONE

# INTRODUCTION AND OBJECTIVES

## 1.1. Introduction

Beta- lactamases are serine enzymes (class A, C, OR D of Amber classification) or metalloenzyme (class b of Amber classification) that confer resistance to β-lactams by hydrolyzing their β-lactam ring (Bush and Jacoby 2010).The discovery and use of new classes of β-lactams have immediately been followed by the emergence of new β-lactamases. The first reported β-lactamases TEM-1/2 and SHV-1, describe since 1960 from *E.coli* and *K. pneumoniae* were usually chromosomal. They are able to inactive penicillins (amoxicillin, ampicillin, ticarcillin) hence the name penicillinase narrow spectrum. From the 80s, plasmid mediated extended – spectrum Beta- lactamases (ESBLs) have been described and confer resistance to penicillins, oxyiminocephalosporins (cefotaxime, ceftazidime, ceftrixone, cefuroxime, cefepime) and monobactam (azteronam) (philippon and Jacoby. 1989).

The Gram negative bacilli especially *Pseudomonas species* and members of Enterobacteriaceae are common cause of infection of many parts of the body they account for more than 50% of all isolates in nosocomial infections (Talaro, 1996).

Infections caused by *E*nterobacteriaceae rods are difficult to manage because of the reduction of therapeutic possibilities, resulting from constantly increasing resistance of these organisms to antibiotics (Pitout, 2008).

The production of ESBLs is one of the most prevalent resistance mechanisms in Gram-negative bacilli. Initially, ESBLs were predominantly described in *K. pneumoniae* and *E. coli* strains, but recently the enzymes were found in other genera of the *E*nterobacteriaceae family (Mathur *et al*., 2002).

ESBL - Producing bacteria exhibit effective hydrolyzing of β-lactam antibiotics including broad – spectrum β- lactam drugs and monobactams Except Cefamycins and beta- lactam inhibitors (Bush, Jacoby, 2010).

The resistance usually depends on expression of bla genes belonging to the inter alia $^{bla}$*TEM*, $^{bla}$*SHV,* and $^{bla}$*CTX-M* genes family. The $^{bla}$*TEM*$^{bla}$*SHV*$^{bla}$*CTX-M* genes are responsible for production of respectively, *TEMβ*-lactamase, *SHVβ*–lactamase, *CTX-MB*-lactamase, large families of Enzymes with evolutionary affinity. Since the first *TEM-1β*-lactamase was discovered, one hundred eighty-five new β-lactamases of the *TEM* family have been reported worldwide, where as ninety –three variants are responsible for production of ESBLs. Among one hundred seventy –two enzyme types of *SHV* family, forty-five have been reported as ESBL.the*CTX-M* family comprises more than sixty enzymes (AL-Jassera, 2006; Shah *et al*., 2012).

## 1.2. Rationale

Resistance to third generation cephalosporin antimicrobial agents become worldwide problem resulting in the failure of treatment and emerging of serious bacterial infection

According to study done in Khartoum hospitals the frequency of ESBL producing isolates are very remarkable 45.1 % (Ibrahim *et al*., 2013).

## 1.3. Objectives

### 1.3.1General objective

To detect Extended Spectrum Beta - lactamase among Gram- negative bacteria isolated from patients in Omdurman Military Hospital.

### 1.3.2 Specific objectives

1-To reidentify clinical isolates

2- To detect the β –lactamase producer bacteria by phenotypic methods

3-To determine the frequency of β-lactamase producing bacteria in association with age group and gender.

# CHAPTER TWO

# LITERATURE REVIEW

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Background

Antibiotics resistance is not recent phenomenon, resistance to penicillin developed in some bacteria as early as 1940, the escape of the problem become apparent in the 1980s and 1990s when scientist and physicians observed treatment failure on a large scale (Cowban *et al*., 2006).

Beta lactamase are enzymes that catalyze the hydrolysis of the beta-lactam ring to yield microbiologically inactive products. Genes encoding these enzymes are wide spread in the bacterial kingdom and are found on the chromosome and on plasmid. The beta- lactamase of Gram- positive bacteria are released into the extracellular environment and resistance will only be manifest when large population of cell is present. The enzymes of Gram –negative cells, however, remain within the periplasm. (Richi *et al*., 2012)

The enzyme can transferred from one bacterium to another mostly by conjugation (except in *Staphylococcus aureus*) where they are transferred by transduction. β- lactamase can be classified into two ways: amblers classification (structural or molecular classification) according to this β-lactamase are classified into four classes. Bush, Jocoby Medeiros classification or functional (phenotypic) classification most advanced and complex classification (Apurba and Sandhya, 2016).

Resistant to β- lactam antibiotics continues to increase, mostly due to the presence of various beta-lactamase. As a result of the ability of the plasmids to acquire additional resistance determinants, many of the beta-lactamase producing pathogens became multidrug resistant. The most important beta-lactamase, which compromise the use of beta-lactamase

new days are extended –spectrum beta-lactamases inhibitor-resistant *TEM* and *SHV* beta-lactamase and Carbapenems. They belong to molecular classes A, B, and D. class A comprises Carbapenemases sensitive to inhibition by clavulanic acid, most of them are chromosomally encoded, but some of them are plasmid- mediated such as *KPC-1* in *K. pneumoniae* and *GES-2* in *P. aeruginosa* the class B Carbapenemases are metallo-beta-lactamase of the *IMP* or *UIM* group. The classes D Carbapenemases are most frequent in *Acinetobacter baumannii* (Li *et al.,* 2005).

Enzyme β-lactamase converts penicillin into harmless penicillin acid. It's probable that the ability to produce penicillinase always has existed in certain bacterial mutants. But that the ability manifests itself when the organism are confronted with the drug. Thus a process of natural selecting takes place, and rapid multiplication of penicillinase- producing bacteria yields organism over which penicillin has no effect (Pommervile, 2004).

### 2.2Resistant in Gram-negative bacteria

Among Gram –negative bacteria, the emergence of resistance to extended- spectrum Cephalosporins has been a major concern. It appeared initially in a limited number of bacterial species *(E. cloacae*, *C. freundii*, *S. marcescens*, and *P. aeruginosa*) that could mutate to hyper produce their chromosomal class C β-lactamase.

A few years later, resistance appeared in bacterial species not naturally producing AmpC enzymes (*K. pneumoniae*, Salmonella spp, *P. mirabilis)* due to the production of *TEM*- or *SHV*-type ESBLs.

Characteristically, such resistance has included oxyimino-(for example ceftizoxim, cefotaxime, ceftrixone and ceftazidime, as well as the oxyimino-monobactamsazteronam), but not 7-alpha methoxyl-

cephalosporins (cephamycins; in other words, cefoxitin and cefotetan); has been blocked by inhibitors such as clavulanate, sulbactam or tazobactam, and did not involve Carbapenems and temocillin. Chromosomal-mediated *AmpC* β-lactamase represent a new threat, since they confer resistance to 7-alpha-methoxyl- cephalosporin's (cephamycins) such as cefoxitin or cefotetan are not affected by commercially available β-lactamase inhibitors, and can, in strains with loss of outer membrane porins, provide resistance to Carbapenems (Philippon *et al*., 2002).

### 2.3 Extended-spectrum beta-lactamase (ESBL)

Member of the family commonly express plasmid –encoded β-lactamase (e.g.*TEM-1*, *TEM-2*, and *SHV-1*). Which confer resistance to penicillin's but not to extended- spectrum cephalosporin. In the mid- 1980s, a new group of enzymes, the extended- spectrum β-lactamase (ESBLs), was detected (first detected in 1979). (Sanders and Sanders, 1972).The prevalence of ESBL- producing bacteria has been gradually increasing in acute care hospitals. (Spadafino *et al*., 2014). ESBLs are β-lactamase that hydrolyzes extended –spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftrixone, and ceftazidime, as well as the oxyimino-monobactamazteronam. Thus ESBLs confer multi-resistance to these antibiotics and related oxyimino-beta- lactams. In typical circumstances, they derive from genes for *TEM-1, TEM-2*, or *SHV-1* by mutations that alter the amino acid configuration around the active site of these β-lactamases. Abroader set of β-lactam antibiotics are susceptible to hydrolysis by these enzymes. An increasing number of ESBLs not of *TEM* or *SHV* lineage have recently been described (Emery and Weymouth, 1997).

The ESBLs are frequently plasmid encoded. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (e.g. amino glycosides). Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited. Carbapenems are the treatment of choice for serious infections due to ESBL-producing organisms, yet carbapenem- resistant (primarily ertapenem resistant) isolate have recently been reported (National Committee of Clinical Standard, 2003).

## 2.4 Types of β-lactamases

### 2.4.1 *TEM* β-lactamases (class A)

*TEM -1* is the most common encountered beta-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Cooksey et al.,1990).

Also responsible for the ampicillin and penicillin resistance that seen in *H. influenzae* and *N. gonorrhoae* .The amino acid substitutions responsible for the ESBL phenotype cluster around the active site of the enzyme and change its configuration, allow access to oxyimino-beta-lactam substrates. Opening the active site to β-lactam substrate also enhance 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. Based upon different combinations of changes, currently 140 *TEM*-type enzymes have been described. *TEM-10*, *TEM-12,* and *TEM-26* are among the most common in the United States (temoniera) (Paterson *et al*., 2003; Bradford, 2001; and Jacoby and Mumoz, 2005).

The term comes from the name of the Athenian patient (temoniera) from which the isolate was recovered in 1963(Ruiz, 2001).

### 2.4.2 *SHV* β-lactamase (class A)

*SHV-1* shares 68 percent of its amino acid with *TEM-1* and has a similar overall structure. The *SHV-1*β- lactamase is most common in *K.pneumoniae* and is responsible for up to 20% of the plasmid-mediated Ampicillin resistance in these species. ESBLs in this family also have amino acid changes around the active site, most commonly at position 238 or 238 and 240. More than 60 *SHV* varieties are known. *SHV-5* and *SHV-12* are among the most common (Paterson *et al*., 2003).

### 2.4.3 *CTX-M* β-lactamase (class A)

These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e.g. cefotaxime, ceftrixone, or cefepime). Rather than arising by mutation, they represent example of plasmid acquisition of β-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms. these enzymes are not very closely related to *TEM* or SHV β-lactamases in that they show only approximately 40% identity with these two commonly isolated β-lactamases. More than 80% *CTX*-Menzymes are currently known. Despite their name, a few are more active on ceftazidime than cefotaxime. They have mainly been found in strains of *Salmonella* enteric serovar*Typhimurium* and *E. coli,* but have also been described in other species of Enterobacteriaceae and are predominant ESBL type in parts of South America. *CTX-M-14*, *CTX-M-3*, and *CTX-M-2* are most widespread.*CTX-M-15* is wide spread in *E. coli* (Woodford et al., 2007). Enzyme beta-lactamase *CTX-M-15*, along with

*ISEcp1*, has been to have recently transposed onto the chromosome of *K. pneumoniae ATCC BAA-2146* (Hudson *et al*., 2014).

## 2.5 Tests used to detect the ESBL

### 2.5.1 Screaning methods

CLSI has developed both disk diffusion and micro dilution screening tests using selected antimicrobial agents. Each *K. pneumaniae*, *K. oxytoca*, or *E. coli* isolate should be considered potential ESBL producer .the sensitivity of screening for ESBLs in enteric organisms can vary depending on which antimicrobial agents are based suggested for screening will improve the sensitivity of detection cefpodoxime and ceftazime show highest sensitivity for ESBL detection (Atlanta, 2010 ;Taneja and sharma, 2008).

### 2.5.2 Confirmatory method disk diffusion method

Mueller Hinton agar plate is taken and inoculated with the test organism then Ceftazidime (30µg) and Cefotaxime (30µg) disk are used, alone and with clavulanic acid (10µg). A $\geq$ 0.5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirm an ESBL producing organism (Mohanty, 2010).

Other combination like pipercillin / Tazobactam (100/ 10µg) and cefoperazome / sulbactam (75/ 30µg) can be used (Afraid and Faroogi, 2012).

### 2.5.3 Double disk diffusion synergy test

The test was performed as described by Jarlier *et al.,* A sterile Mueller-Hinton Agar was prepared and a 0.5 McFarland equivalent standard of the test organism was streaked on the surface of the Agar with a sterile loop and allowed for 15-20 minute to pre-diffuse. An augmentin which is a combination of clavulanic acid (20µg) and amoxicillin (10µg) was placed at the centre of the plate and cefotaxime (30µg) ceftazidime (30µg) were placed 15mm apart centre to centre on the plate with sterile forceps. These were incubated at 35ºc for 18-24hr. and enhanced zone of inhibition from 5mm above in the presence of augmentin is regarded as positive for phenotypic production of ESBL enzyme (ALsalam, 2012; Ngozi, 2010).

#### 2.5.4.1 E test ESBL strips.

This combines both the principle of dilution and diffusion techniques. E strip is a thin nonporous plastic strip 5mm wide and 60mm long it carries two shorter gradients aligned in opposing direction on a single strip. One end generates a stable concentration gradient of one of the oxyiminocephalosporins (e.g. ceftazidime), while other end generates a gradient of cephalosporin+ clavulanic acid (4µg/ml). When the E strip is applied over a lawn culture inhibition ellipse may be seen on the both ends of the strip. MIC is interpreted as the point of intersection of the inhibition ellipse with E test strip edge. This method is more sensitive and easy to use (Revathi, 1997).

### 2.5.5 Combined disc methods.

These depend on comparing the zone given by disc containing an extended-spectrum cephalosporin with clavulanate with those for identical discs without this inhibitor. If an ESBL is present, the zones are enlarged by the presence of the inhibitor. The United State Clinical Laboratory Standards Institute (CLSI; formerly) recommends comparison of zones given by cefotaxime (30 µg) versus cefotaxime +clavulanate (30+10µg0 and ceftazidime (30µg) versus ceftazidime + clavulanate (30 +10µg) discs (National Committee of Clinical Standard, 2003).

### 2.5.6 Automated susceptibility testing systems.

It should be noted that ESBL detection tests are incorporated into standard test for Vitek and Vitek 2 (bioMerieux, Marcy I'Etiole, France) and the panels for Phoenix (Becton Dickinson). The Vitek 2 differs in that, rather than incorporating a specific ESBL test, it best- fits the patterns of MICs profiles for isolate to those of a reference database of MICs for isolates with known mechanisms of resistance, including ESBLs .TheVitek ESBL test was *CTX-M* enzymes were recognized, as well as those with *TEM* and *SHV*- derived enzymes, the only errors being a few K1 β-lactamase hyper producers that were misidentified as ESBL-positive (CLSI, 2011).

### 2.5.7 Reference method

The reference method was MIC by agar dilution technique performed in accordance with CLSI guidelines. The MIC test was done on all the isolates. the agar dilution method was performed with Mueller-Hinton agar plates containing serial twofold dilution of cefotaxime, ceftazidime, and cefepime at concentration ranging from 0.25 to 512µg/ml, with and without clavulanic acid at fixed concentration of 4µg/ml. each bacterial suspension was inoculated as spots with wire loop calibrated to deliver 0.001ml spread over a small area and incubated at 37°c for 18 to 24 hours. The test was positive if a ≥ 3 twofold reduction was observed in

the MIC of the cephalosporin combined with clavulanic acid compared with the MIC of the cephalosporin alone (CLSI, 2011).

### 2.5.8 Vitek 2 compact system (bioMerieux, Marcy 1`Etoile, France)

Vitek 2 compact is an integrated system that automatically performs rapid identification using algorithms based on fluorescence and colorimetry, and antimicrobial susceptibility testing (AST) based on kinetic analysis of growth data. It features an advanced expert system (AES) that interprets the antibiotic resistance phenotype. A Vitek card for susceptibility testing (AST-GN25), containing ESBL confirming test panel, was inoculated and incubated following the manufacturer's recommendation. An isolate was considered ESBL positive if the phenotypic by the AES included ESBL with or without decreased outer membrane permeability (i.e., porin loss) and negative if only the wild type or B-lactamase other than ESBLs were proposed by AES. All other interpretation results were considered indeterminate (Perez-Vazquez *et al*., 2001).

### 2.5.9. Molecular characterization of ESBL producing –isolate

All isolates which were positive for synergy test screened in order to detect B-lactamase encoding genes for $^{bla}$*TEM*, *SHV* and *CTX-M* families by previously described PCR protocols (Olivir *et al*., 2002; pagari *et al*., 2003). DNA template was prepared from purified bacteria grown overnight at 37ºC.

On Mueller Hinton Agar plate crude DNA extraction were obtained by suspending a colony in 100 ml of purified water and boiling at 95ºC for 10 mn (Munday *et al*., 2004). Mixing PCR for one reaction has the following component: 5 ml of green buffer 5X; 1ml of each primer 10µm; 0.65µl of dNTPs 10µm; 0.12µl of Gotag 0.5u/µl and 15.25µl of purified water. Reaction volume is completed to 25 µl with 2µl of DNA template:

primers supplied by pro mega according to each B-lactamase gene type. PCR was carried out under the following conditions on senoQestlabcyclar, GmbH Germany; Initial denaturation step at 96ºc for 5 min, followed by 35 cycle consisting of denaturation at 96ºc for 1min, annealing at 58ºC, 60ºC and 50ºC for *TEM, SHV*, *CTX-M* at 1 min, primer extension at 72ºC for 1min and final Extension for 10 min. migration of PCR products watched by green buffer (Eugentec) during 30 min at 80 V was performed on agar gel of 1% prepared with BET at 0.25% as final concentration (Mundy *et al*., 2004)

## 2.6. Treatment and control of ESBL

The epidemiology of these infections is often complex; multiple colonal strains causing focal outbreaks may co-exist with sporadic ones. Relevant infection-control measures should focus on reducing patient- to-patient transmission via the inanimate environment, hospital personnel, and medical equipment. Wise use of antibiotics is also essential. The available therapeutic options for the treatment of ESBL-associated infections are limited by drug resistance conferred by the ESBLs, along with frequently observed co-resistance to various antibiotics classes including Cephamycins, Fluoroguinolones, Aminoglycosides, Tetracyclines, and Trimethoprim/ Silfamethoxazole. Relevant clinical data regarding the effectiveness of different regimens for ESBL-associated infections are limited. Although certain cephalosporins may appear active *in vitro*, associated clinical outcomes are often suboptimal. B-lactam/ β-lactam inhibitor combination may be of value, but the supporting evidence is weak. Carbapenems are regarded as the agent of choice, and may be more effective than fluoroguinolones for serious infections. Tigecycline and polymyxins have substantial antimicrobial activity against ESBL-producing Enterobacteriaceae, and, along with Fosfomycin. Merit further evalution (Falagas, 2009).

# PREVIOUS STUDIES

Ibrahim *et al*., (2013) reported a total of 232.*E. coli* isolates were collected from various clinical specimens of patients' indifferent hospitals in Khartoum State, Sudan. Isolates were identified, and tested for The antimicrobial susceptibility and screened for ESBL production as standard methods. the double disk diffusion method was used to confirm ESBL production using antimicrobial disks of Ceftazidime 30(µg), Cefotaxime 30(µg), with or without clavulanic Acid 10(µg). Azone Difference of > 5mm between disks was considered indicative of ESBL Production.

Out of 232 *E. coli* isolates, 70(30, 2%) were found to be positive for ESBL by the applied phenotypic methods. ESBL producing isolates yielded high resistance rate for Trimethoprim- Sulfamethaxazole (98.6%)Tetracycline (88.6%), Nalidixic Acid (81.4%) and Ciprofloxacin (81.4%). The higher antimicrobial activites of ESBL –producing isolates were Observed for Amikacin (95.7%) followed by Tobramicin (74.3%) and Nitrofurantion (68.6%). Resistance to Quinolones, Aminoglycosides, Trimethoprim-Sulfamethoxazole, Tetracycline, Nitrofurantion and Chloramphenicol was higher in ESBL than non ESBL isolates ($p<0.05$). The frequency of ESBL- producing isolates varied among Hospitals (18.2% - 45.1%). Although higher prevalence was recorded as 45.1% at Khartoum Teaching Hospitals. Wound specimens were the most Common source of ESBL- producing isolates. The proportion of ESBL-Producing *E. coli* did not differ significantly between adults and children (31% vs. 27%).

The prevalence of ESBL- producing *E. coli* detected in this study is of Great concern, which requires infection control measures including Antimicrobial management and detection of ESBL –producing isolates.

Out of 70 urine specimens, 56(56%) of samples gave significant Bacterial growth. The Gram negative bacilli were 42(75%) the most Frequent Gram- negative were *K. oxytoca* 15(26.8%) and *E. coli* 10(17%),While the most abundant Gram- positive was *S. saprophyticus* in the Susceptibility test, 5 isolates were resistant to 3$^{rd}$ generation Cephalosporin Ceftazidime, Ceftrixone and Cefotaxime as follow: 2 *K. pneumoniae,* 2 *E. coli,* and 1 *P. vulgaris*. ESBL production test done for all bacterial resistance to 3$^{rd}$generation cephalosporin and then Confirm by combination test using Double Disc synergy test 5(11.9%) Isolates gave positive result. The PCR was done for 5 isolates using *TEM*, *SHV*, and *CTX-M* primers. *TEM* gene was the most predominant one Followed by *CTX-M* and the one was *SHV* gene. From these results it could be concluded that these genes are responsible for the resistance of Cephalosporins (Nosaiba and Yousif. 2014).

# CHAPTER THREE

# MATERIALS AND METHO

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study design

### 3.1.1 Type of study

This was retrospective study conducted to detect *TEM*, *SHV*, and *CTX-M* genes in bacteria isolated from patients.

### 3.1.2 Study area

This study was done in Military Hospital.

### 3.1.3 Study duration

The study was carried out in the period from October to November, 2018.

### 3.1.4 Source of bacterial isolates

Isolates were obtained from microbiology laboratory previously isolated from urine, pus, wound.

## 3.2 Sample size

Total of 70 bacterial isolates were used in this study.

## 3.3 Data collection

A predestined form was used for collection of sociodemographic data

## 3.4 Sample collection

The isolates were previously isolated from patients by using standard microbiological procedures. Pus from wound was collected by sterile cotton Swab, urine in wide mouthed container and sputum in wide mouthed Container. All specimens were transported to microbiology

laboratory of the hospital with minimum delay for culture and sensitivity test.

### 3.5 Ethical consideration

This study was approved by The College Ethics Committee, College of Medical Laboratory Science, and SUST after explaining the study and it is goal. An informed consent from head of Microbiology Department of Omdurman military hospital.

### 3.8 Laboratory work

#### 3.8.1 Checking the purity of the isolates

The isolates were streaked on nutrient agar and inoculated over night at 37C°. At the end of incubation period, a discrete colony was picked up and checked for purity under microscope; then stored in Bijon bottle containing nutrient agar slant for further investigation.

#### 3.8.2 Re- identification of the isolate

##### 3.8.2.1 Gram stain

Bacterial smear was prepared by transferring portion of discrete colony to drop of normal saline. The smear was covered with crystal violet stain for 30-60 second, rapid washed the stain with clear water, then smear was covered with lougou's iodine for 30-60 second, washed of the iodine with clear water, decolorized rapidly (few, second) with acetone- alcohol, washed immediately with clear water, then the smear was covered with safranin for minute, washed of the stain with clean water, wipe back of the clean and placed in the draining rack for the smear to dry air. The smear was examined microbiologically with oil immersion objective to report bacterial Gram stain reaction and shape gram -negative rod bacteria; stain pink red (Cheesbrough, 1989).

#### 3.8.2.3 Biochemical tests

##### 3.8.2.3.1 Indol test

In this test the tested organism produces tryptophanase which break down tryptophan and produce Indol, which react with kovac's reagent and give pink ring. The tested organism was inoculated into peptone water and incubated at 37Cº for overnight, kovac's reagent was added. If there pink ring the result was indicated as positive there is no pink ring in surface the result was indicated as negative (Cheesbrough, 1989).

##### 3.8.2.3.2 Citrate utilization test

This test is one of several techniques used occasionally to assist in the identification of enterobacteriaceae. The test is based on ability of an organism to use citrate as its only source of carbon. dense bacterial suspension of the test organism was prepared in 0.25 ml sterile physical saline in small tube, a citrate tablet and stopper the tube were added, incubate overnight at 37Cº.red colour positive test and yellow orange colour negative citrate test (Cheesbrough, 1989).

##### 3.8.2.3.3. Urease test

Testing for Urease enzyme activity is important in differentiating on *Proteus* strains are strong Urease producers. *Y. enterocolitica* also shows Urease activity (weakly at 35-37Cº). *Salmonellae* and *Shigellae* do not produce Urease. The test organism is cultured in a medium which urea and the indicator phenol red. When the strain is Urease producer. The enzyme will break down the urea (by hydrolysis) to give ammonia and carbon dioxide. With the release of ammonia, the medium become

alkaline as shown by a change in colour of indictor to pink (Cheesbrough, 1989).

#### 3.8.2.3.4 KIA reaction

This reaction is used to help identify salmonellae following isolation on primary selective medium. KIA reaction is based on the fermentation of lactose and glucose and the production of hydrogen sulphide. Straight wire was used to inoculate KIA medium first stabbing the butt and then streaking the slope in azig-zig pattern and overnight incubation at 37Cº.then the reaction read (Cheesbrough, 2000).

### 3.8.2.4 Antibiotic susceptibility testing and ESBL detection

Antibiotics susceptibility was performed by disk diffusion method with antibiotics disk to test Gram- negative bacilli particularly monobactams: azteronam (30µg), the third generation cephalosporin like Cefotaxime (30µg). Ceftrixone (30µg) Cefotaxime (30µg) Ceftazidime (30µg) and forth generation cephalosporin; Cefepime (30µg). Antibiotics were tested on Petri dish containing Muller Hinton agar. Measurement of inhibition zone determines the clinical categories (CA-SFM, 2010, 2011; 2012) isolate that is resistant to at least one of the antibiotics in clinical test, using NCCLs method (NCCLs,2000) were collected, purified and conserved at -80Cº.

## 3.8.3 Confirmatory test

### 3.8.3.1 Double disc diffusion synergy test

A plate was inoculated with the test isolate, as for a routine British Society for Antimicrobial Chemotherapy (BSAC) disc susceptibility test. Discs containing Cefotaxime and Ceftazidime 30µg were applied to the medium either side of one containing Co-amoxiclav 20+10 µg; and 25-30

mm away from it and the plate was incubated overnight at 37Cº, and ESBL production is inferred when the zone of either cephalosporin is expanded by the clavulanate. For *CTX-M* type, the synergy is more obvious with Cefotaxime whilst, for whom *TEM* and *SHV* variants, it is more obvious with Ceftazidime. The advantage of this simple method the low cost; the disadvantage is that the optimal separation of discs may vary with the individual strain. Consequently isolates giving equivocal results may need to be re-tested with the discs closer together. Double disc tests between Cefotaxime and co-amoxiclav discs can be performed similarly, and to detect both the *TEM* / *SHV* and *CTX-M* type enzyme. (NCCLs, 2000)

### 3.8.3.2. Combination disks test

Standardized inoculums of bacterial suspension equivalent to 0.5 McFarland standard turbidity of each isolate was inoculated on Muller Hinton agar pates by using a sterile cotton swab. Then with sterile forceps the disk of Ceftazidime and Ceftazidime +clavulanate were placed. After incubation at 37°C for 18 hours aerobically. If a ESBLs is present the zone are enlarged by the presence of inhibitor (CLSI, 2006).

### 3.8. 3.4Data analysis

Data were computed and analyzed by using statistical package for social science (SPSS) computer software version 16 to check the statistical Significance the *P*-value considered significant was $< 0.05$.

### Quality control

*K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as ESBL positive and negative controls, respectively (CLSI, 2011).

# CHAPTER FOUR

# RESULTS

# CHAPTER FOUR

# RESULTS

Clinical isolates 70 were collected from Military Hospital. The bacterial isolates were recovered from 25(35,7%) female and 45(64,3%) male that show in (Table 1). the age of participants range from 2 - 89 years as in (Table 2). The largest number of isolates 59(59%) were recovered from patients aged 31- 60 years while the lowest number of specimens 16 (16%) were recovered from patients aged 31-90 years seen in (Table 2).

Cultivation of specimens on CLED agar, MaCconkey agar and blood agar, gave bacterial growth in 70 clinical specimens. The isolates were reidentified by their colonial morphology, Gram stain and biochemical tests. The bacteria that identified were 38 bacteria from urine, 14 bacteria from wound and 18 bacteria from sputum. The *E. coli* is the commonest bacteria isolates was 30(42%) among urine specimens, while *K. pneumoniae* the commonest bacteria in sputum showed in (Table 3). *E. coli* is the most bacteria that show producing of ESBLs as showed in (Table 6). The detection of ESBL by confirming test, ESBL screening test 28, 5% is negative result and 71, 4% is positive. ESBL DDST method showed78, 5 is negative ESBL and 21, 4 is ESBL positive result. Combination test negative result is 78, 5 and 21, 4 positive show in (Table 4). the frequency of bacteria isolated from males patients compared with that isolated from females patients was found insignificant (p=0.696) in (Table 7). The frequency of ESBLs producer in different age groups was found insignificant (p=0.195) as showed in (Table 8).

**Table 1. Distribution of clinical isolates according to the gender**

| gender | Source of isolates | | | Total |
|---|---|---|---|---|
| | Wound swab | Sputum | Urine | |
| male | 10 | 10 | 25 | 45 |
| female | 4 | 8 | 13 | 25 |
| Total | 14 | 18 | 38 | 70 |

**Table 2. Distribution of clinical specimens according to the age groups**

| Age group | Source of isolates | | | Total |
|---|---|---|---|---|
| | Wound swab | Sputum | Urine | |
| 1-30 | 6 | 6 | 8 | 20 |
| 31-60 | 8 | 10 | 20 | 38 |
| 61-90 | 0 | 2 | 10 | 12 |
| Total | 14 | 18 | 38 | 70 |

**Table 3. Distribution of organisms isolated according to type of specimens**

| Isolate organism | Source of isolates | | | Total |
|---|---|---|---|---|
| | Wound a swab | Sputum | Urine | |
| *E. coli* | 10 | 0 | 30 | 40 |
| *K. pneumoniae* | 0 | 18 | 2 | 20 |
| *P. aeruginosa* | 4 | 0 | 4 | 8 |
| *Proteus* ssp | 0 | 0 | 2 | 2 |
| Total | 14 | 18 | 38 | 70 |

**Table 4. Detection of ESBLs by confirmatory test**

| Test | Negative ESBL | | Positive ESBL | | Total |
|---|---|---|---|---|---|
| | % | NO | % | NO | |
| ESBL screening | 28,5 | 20 | 71,4 | 50 | 70 |
| ESBL DDST | 78,5 | 55 | 21,4 | 15 | 70 |
| Combination test | 78,5 | 55 | 21,4 | 15 | 70 |

**Table 5. Frequency and percentage of ESBLs producers and non ESBLs producers isolates**

| **Type of specimens** | **ESBLs** | | **Total %** |
|---|---|---|---|
| | Negative % | Positive % | |
| Urine | 25(35, 7%) | 13(18, 5%) | 38(54, 2%) |
| Wound swab | 12(17, 1%) | 2 (2, 8%) | 14(20.0%) |
| Sputum | 18(25, 7%) | 0(0.0%) | 18(25, 7%) |
| Total | 55(78, 5%) | 15(21, 4%) | 70(100%) |

(***p*. value=0.011**)

**Table 6. Frequency and percentage of ESBLs producer and non ESBLs producers isolates among organism**

| **Organism** | **ESBLs** | | **Total %** |
|---|---|---|---|
| | Negative % | Positive % | |
| *E. coli* | 26(37.0%) | 14(20.0%) | 40(57, 0%) |
| *K. pneumoniae* | 19(27, 1%) | 1(1, 4%) | 20(28, 5%) |
| *P. aeuroginosa* | 8(11, 4%) | 0(0.0%) | 8(11, 4%) |
| *Proteus* ssp | 2(2, 8%) | 0(0.0%) | 2(2, 8%) |
| Total | 55(78.6%) | 15(21, 4%) | 70(100%) |

(***P*. value=0.001**)

**Table 7. Frequency and percentage of ESBLs producers and non producers isolates among gender**

| Gender | ESBLs | | Total % |
|---|---|---|---|
| | Negative % | Positive % | |
| Male | 36(51, 4%) | 9(12, 8%) | 45(64 3%) |
| Female | 19(27, 1%) | 6(8, 6%) | 25(35, 7%) |
| Total | 55(78, 6%) | 15(21, 4%) | 70(100%) |

**(*P* .value = 0.696)**

**Table 8. Frequency of ESBLs producers among age group**

| Age group | ESBLs | | Total % |
|---|---|---|---|
| | Negative % | Positive % | |
| 1-30years | 20(28, 6%) | 5(7, 1%) | 25(35, 7%) |
| 31-60years | 23(32, 8%) | 7(10, 0%) | 30(42, 8%) |
| 61-90 years | 12(17, 1%) | 3(4, 3%) | 15(21, 4%) |
| Total | 55(78, 6%) | 15(21, 4%) | 70(100%) |

**(*P*.value=0.945)**

**Table 9: Antimicrobial susceptibility pattern of isolated bacteria**

| Antibiotics | *Proteus spp* | | *P. aeruginosa* | | *K. pneumoniae* | | *E. coli* | |
|---|---|---|---|---|---|---|---|---|
| | R | S | R | S | R | S | R | S |
| Cefotaxime | 0<br>0% | 2<br>2.8% | 3<br>4.2% | 5<br>7.1% | 10<br>14.2% | 10<br>14.2% | 10<br>14.2% | 30<br>24.8% |
| Ceftrixone | 1<br>1.4% | 1<br>1.4% | 1<br>1.4% | 7<br>10.0% | 15<br>21.4% | 5<br>7.1% | 5<br>7.1% | 35<br>50% |
| Cefepime | 1<br>1.4% | 1<br>1.4% | 0 | 8<br>11.4% | 2<br>2.8% | 18<br>25.7% | 25<br>35.7% | 15<br>21.4% |
| Ceftazidime | 1<br>1.4% | 1<br>1.4% | 0 | 8<br>11.4% | 0 | 20<br>28.5% | 20<br>28.5% | 20<br>28.5% |
| Azteronam | 0<br>0% | 2<br>2.8% | 2<br>2.8% | 6<br>8.5% | 0 | 20<br>28.5% | 0 | 40<br>57.1% |

# CHAPTER FIVE

## Discussion

# CHAPTER FIVE

## 5-Discussion

### 5.1-Discussion

Nowadays, resistance to antibiotics is a real public health care concern worldwide. It has been clearly demonstrated that the production of B-lactamase is the most important mechanism of antibiotics resistance in Gram negative bacteria. (Diagbouga *et al*., 2016). In the present study, ESBL- producers were detected phenotypically by DDST and combined disc method .based on the results of this study out of 70different clinical specimens examined, 70 gave bacterial growth. In this study 15(21.4%) isolates producing ESBL, the ratio of ESBL producing bacteria was found to be (21.4%) this figure is low compared to the figure in another study done by (Abd. ELRahman. Abd ELRahman. 2017) in Port Sudan Teaching Hospital recorded ESBL- producing isolates( 44.4%) the distribution of ESBL among *E. coli*, *K. pneumoniae, P. aeruginosa*, and *Proteus* spp was (20%) (1.4%) (0%) (0 %). This result was agreed to Omer (2013) in Sudan and MekkiA. Hassan A and M Elsayed. (2010) reported that *E. coli* and *Klebsiella* spp are the most ESBL producers .In the current study urine was the main source of ESBL producing isolates (87%) which is in close agreement with study done by Akbar *et al.,* (2007) (70.4%) This result is due to high ratio of *E. coli* among UTI patients (causing 60%cases) than other samples (Cheesbrough, 1989). By another way this results disagreed with a study done in other countries India by Rudrech and Nagarathnamma (2011) where (70%) of ESBLs producing Isolates were obtained from exudates. In current study the ESBL producers isolated from males (12.8%) and females (8.6%).These findings are lower than result obtained by Almugdam *et al.,* (2016) who

reported ESBLs producers were 30 % in males and 38 % in females. The present study shows insignificant difference between ESBL producing and gender (*P*. value 0.696) this result is in agreement with previous study reported by Aknabi. (2013).

In this study there was no significant difference observed in ESBL-producing isolates among age group enrolled the study (*P*. value 0.945) a previous study by Moyo *et al.,(*2010) reported significant higher ESBL-producing in isolates from children rather than adults, in my study the ESBL- producing isolate from adults rather than children due to large specimens obtained from adult .

## 5.2. Conclusion

The study concluded that there is high ratio of ESBLs among isolated Bacteria in Omdurman Military Hospital. The major organism produces ESBLs is *E. coli.*

## 5.3 Recommendations

1- Detection of ESBL producer should be introduced as routine tests in Microbiology labs for rapid detection of resistant isolates and controls their spread.

2- A larger sample size should be obtained from many hospitals to give more reliable and accurate results.

3- Advanced techniques should be used such as PCR for detect the other ESBLs genes.

4- To avoid the disadvantage of the phenotypic methods of ESBL detection

5- Prevention and control program of antibiotic resistance should be preformed to control the spread of ESBL producer bacteria.

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# Appendices

Questionnaire

-Patient code

-Type of sample

Urine(  )   wound swab (  )   sputum (  )

-Sex

Female (  )   male (  )

-Age

1-30 years (  )

31-60 years (  )

61-90 years (  )

Residence

-Job

-Take antibiotics before 3 months

Yes (  )   No (  )