بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Detection of- β lactamases Wide Spectrum Among Bacteria Isolated from Patients in Military Hospital, Omdurman

الكشف عن انزيمات البيتا لاكتام واسعة الطيف في البكتريا المعزولة من المرضى في المستشفى العسكري،أمدرمان

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الآية

قال تعالى :

{{ يَا أَيُّهَا النَّاس إِن كُنتُم فِي رَيبٍ مِن البَعث فَإِنَّا خَلَقنَاكُم مِن تُرَابٍ ثُمّ مِن نُطفَةٍ ثُمّ مِن عَلَقَةٍ ثُمّ مِن مُضغَةٍ مُخَلَّقَةٍ وغَيرِ مُخَلَّقَةٍ لِنُبَيِّنَ لَكُم وَنَقِرَّ فِي الأرحَام مَا نَشَاء إِلَى أَجَلٍ مُسَمَّى ثُمَّ نُخرِجُكُم طِفلاً ثُمَّ لِتَبلُغُوا أَشَدَّكُم وَمَنكُم مَن يَتَوَفَّى وَمِنكُم مَن يُرَدُّ إِلَى أرذَلِ العُمُر لِكَيلاً يَعلَمَ مِن بَعدِ عِلمٍ شَيئًا وَتَرَى الأرضَ هَامِدَةً فَإِذَا أنزَلنَا عَلَيهَا المَاءَ اهتَزَّت وَرَبَت وَأنبَتَت مِن كُلِّ زوجٍ بَهِيج }}

صدق الله العظيم

سورة الحج : الآية رقم (5)

I

Dedication

To my parents and my sisters.

To my friends.

ACKNOWLDGEMENT

Abbreviations

3GCs	3rd Generation Cephalosporin
CAZ	Cefotaxime
CLED	Cystine Lactose Electrolyte Deficient
CLSI	Clinical and Laboratory Standards Institute
CTX	Ceftazidime
CTX-M	Cefotaximases
E. coli	*Escherichia coli*
DDST	Double disk synergy test
ESBL	Extended Spectrum beta-lactamase
PBPs	Penicillin binding proteins
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SHV	Sulph hydryl variable
SPSS	Statistical Package for Social Sciences
TEM	Temoneira
UTI	Urinary tract infection

ABSTRACT

The emergences of extended-spectrum beta-lactamases (ESBLs) producing bacteria have important clinical and therapeutic implications. The present study was undertaken to detect extended spectrum beta-lactamases among bacteria isolated from different clinical specimens. This study was carried out during period from April to August 2018.

A total of 100 specimens were collected from patients admitted to Omdurman Military Hospital. These were from urine 73(73%), wound swab 20(20%) and sputum 7(7%). The specimens were collected from both males 49 (49%) and females 51 (51%). The ages of participants were range from3-88years. The specimens were cultured on MacConkey agar, CLED agar and blood agar. The isolates were identified by their colonial morphology, Gram stain and biochemical tests. Identified bacteria were subjected to antimicrobial sensitivity test. The antibiotics used were: Gentamicin, Imepnem, Cipofloxcin, Ceftazidime, Amikacin and Norofloxacin. The presence of ESBLs production was detected by double disk synergy and combination disc tests. The results revealed that 65(65%) of the specimens gave bacterial growth, while 35(35%) showed no bacterial growth. 38(58.5%) of bacteria were isolated from urine, 20 (30.8%) from wound swabs and 7 (10.8%) from sputum. The identified bacterial isolates were *K. pneumoniae* 28 (43.1%),

E. coli 27 (41.5%), *P. aeruginosa* 7 (10.8%), and *Proteus* spp 3 (4.6%). ESBLs were detected in 12(18.5%) bacterial isolates. These were 11(16.9%) *E. coli* and 1(1.5%) *K. pneumoniae*. The positive ESBLs producers 5(7.7%) were obtained from males and 7(10.8%) from females. The frequency of ESBLs producers among the groups were2(3.1%) in youth 4(6.2%) in adults and 6 (9.2%) in elderly, 4(6.2%).

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

المستخلص

إن ظهور البكتيريا المنتجة لإنزيمات بيتا لاكتام الممتدة الطيف له تأثيرات سريرية وعلاجية مهمة. هذه الدراسة أجريت بغرض تحديد انتشار إنزيمات بيتا لاكتاماز الممتدة الطيف بين البكتيريا المعزولة من عينات سريرية مختلفة. أجريت الدراسة خلال الفترة من إبريل الى اغسطس 2018 .

جمعت 100 عينة من المرضى في مستشفى السلاح الطبي، شملت العينات (73%)73البول و (20%)20مسحات الجروح و(7%)7 القشع. العينات جمعت من كلا الجنسين الذكور (49%)49 والاناث (51%)51 , وكانت اعمارهم تتراوح ما بين 88-3سنة.

استزرعت العينات في أوساط غذائية شملت ماكونكي، الكليد واجار الدم . تم التعرف على البكتيريا المعزولة بدراسة الشكل الظاهري للمستعمرات وصباغة جرام والاختبارات الكيموحيوية و اجري اختبار حساسية البكتيريا للمضادات الحيوية. المضادات الحيوية التي استخدمت هي الجنتاميسين ،الايميبينيم، السبروفلوكساسين، السفتزيديم، الامايكسين والنوروفلوكساسين. ومن ثم تم للكشف عن إنزيمات الطيف الممتد للبيتالاكتامات باستخدام اختبار تآزر القرص المزدوج و اختبارالقرص المشترك .

أظهرت النتائج أن 65(65%) من العينات اعطت نموا بكتيريا 35 (35 %) من العينات لم تعط،38(58.5%) من البكتيريا المعزولة تم عزلها من البول ،20 (30.8%) من مسحات الجروح و7(10.8%) من القشع. والبكتيريا التي تم التعرف عليها هي: العصوية اللولبية 27 (41.5%) والكلبيسلا 28(43.1%) والزائفة الزنجارية 7(10.8%) والبكتيريا المتقلبة 3 (4.6%).

12 (18.5%) بكتيريا اعطت نتيجة موجبه لإنزيم بيتا لاكتاماز الممتدة الطيف. 11(16.9%)البكتيريا اللولبية و1(1.5%)الكلبسيلا عن طريق اختبار اختبار تآزر القرص المزدوج و اختبارالقرص المشترك . نسبة النتيجة الموجبة للأنزيم التي تم الحصول عليها من الذكور 5(7.7%) بينما الاناث7(10.8%). وكان تكرار النتيجة الايجايية للانزيم بين الفئات العمرية الشباب اليافعين 2(3.1%), الشباب4(6.2%) وكبار السن6(9.2%) خلصت الدراسة الى الانتشار واسع لإنزيم البيتا لاكتام الممتدة الطيف بين البكتيريا المعزولة من مستشفى السلاح الطبي واكثر البكتيريا المنتجة للانزيم هي البكتيريا العصوية. اوصت الدراسة باجراء مزيد من الدراسات بزيادة عدد العينات واستخدام طرق تشخيصية متقدمة للتاكد من صدقية هذه الدراسة.

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CHAPTER ONE
INTRODUCTON AND OBJECTIVES

1.1. Introduction

Serious outbreak of disease caused by Gram-negative rods resistant to antibiotics have occurred in many developing countries (Livenson, 2014). β- lactamases are a large family of hydrolyases that catalyze the hydrolysis of the amide bond in the β - lactam ring of penicillin and cephalosporin (Hall and Barlow, 2003). β– lactamase produced by *Staphylococcus aureus* (*S.auerus*), *Heamophilus* spp, *Escherichia coli* (*E. coli*) and *Klebsiellia* sp. Other β - lactamase like AMPc β-lactamases produced by *Pseudomonas aeruginsoa* (*P.aerginosa*) and *Enterobacter* spp. (Shah *et al*., 2004). Most common type of β– lactamase is Extended Spectrum β– lactamase (ESBL) (Pai *et al*., 2007). Conferring bacterial resistance to the penicillins, third generation of cephalosporins and aztreonam by hyrolysing this antibiotic. ESBLs was first detected in *Klebsellia* spp and then later identified in *E. coli* and other species of enterobacteriacae (Chong and Kamimura, 2011).The ESBLs genes are predominantly plasmid encoded, which can be divided into three genotypes: TEM, SHV and CTX-M (Malloy and Campos, 2011).

1.2. Rationale

Antimicrobial resistance is a growing threat worldwide. Increasing resistance to third generation Cephalosporin has become a cause for concern among bacteria. The ratio of Extended Spectrum β-lactamases (ESBLs) among bacteria constitutes a serious threat to current β– lactam therapy leading to treatment failure. Although a lot of studies were done in Sudan about ESBL, such as Mekki *et al* (2010) and Omer (2013), but resistance increased continuously to traditionally used antibiotics.

1.3. Objectives
1.3.1. General objective

To detect ESBLs producing bacteria isolated from different clinical specimens in Omdurman Military Hospital. This study is expected to screen one the largest hospital in the Omdurman area for presence of ESBLs-producing bacteria.

1.3.2. Specific objectives

1- To isolate and identify bacteria from different clinical specimens.

2- To perform antimicrobials susceptibility test.

3- To determine presence of ESBLs using double–disk synergy test and combination test.

CHAPTER TWO
LITERATURE REVIEW

2.1. β-lactam antibiotics

The β-lactam antibiotics are broad class of antibiotics consisting of agents that contain a β-lactam ring in their molecular structure. These include penicillins, cephalosporins, monobactams and carbapenems. β-lactam antibiotics work by inhibiting cell wall biosynthesis in the bacteria and are the most widely used group of antibiotic (Naas and Normadnn, 1999). β-lactamase is a secreted enzyme that hydrolyzes penicillin and other penicillnase-susceptible compounds into inactive penicilloic acid (Slama, 2008). Other β-lactamase like AmpC β-lactamase produced by *P. aeruginosa* and *Enterobater* spp. the resistant organisms produce penicillin binding protein (PBPs) that have low affinity for binding β-lactam antibiotic. Impaired penetration of antibiotic to target PBPs occurs only in Gram–negative species because of their impermeable outer cell wall membrane which is absent in Gram-positive bacteria (Yasmin, 2012).

2.2 Classification of β-lactamases
2.2.1. Class A: Extended spectrum β-lactamase (ESBLs).

Organisms producing ESBL enzymes are resistant to all penicillins and 1st, 2st and3rd generation cephalosporins and monobactam, however remain sensitive to carbapenems and cephamycins. EBSL has different types TEM, SHV and CTX-M (Bradford, 2001).

2.2.2. Class B: Metallobetalactamase (MBL).

Class B Metallo- β- lactamase (MBLs) have a broad substrate spectrum can catalyze the hyrdolysis of virtually all β-lactam antibiotics. These classes of enzymes are resistant to inactivation by clavulate, sulbactam aztreonam and carbapenem (Lakshmi *et al*, 2014).

2.2.3. Class C: AmpC– β –lactamase.

AmpC type-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 it's 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 plasmid (Helfand and Bonomo, 2003).

2.2.4. Class D:Oxacillin

 Oxacillin (OXA) are a group of β-lactamases occur mainly in *Acinetobater* species and are divided into two clusters. OXA carbpenemases hydrolyses carbapenems very slowly in vitro and the high MICs seen for some *Acinobacter* (>6mg/L) may reflect secondary mechanisms. They are sometimes augmented in clinical isolates by

additional resistance mechanisms, such as impermeability or efflux (Carattoli, 2009).

2.3. Mechanism of resistance

β-lactam antibiotics perform their action by binding to the PBPs, thus inhibiting the synthesis of peptidoglycan. Inhibition of PBPs weakens the bacterial cell wall, resulting in cell growth inhibition and eventually leading to cell death. There are three main mechanisms of β-lactam resistance; decreased access to the PBPs, decreased PBP binding affinity, and destruction of antibiotic by the expression of β-lactamase which can bind and hydrolyse β-lactams (Ambler, 1980).

2.4. ESBL

Extended Spectrum Beta Lactamases are a type of enzymes that mediate resistance to extended –spectrum (third generation) cephalosporins (e.g cefotaxime, ceftazidime and ceftriaxone) and monobactams (e.g aztreonam) but do not affect cephamycins or carbapenem. The most organisms produce ESBL are *E. coli* and *Klebsiella* also other isolates of enterobateriacae, such as *Proteus mirablis* and *P. aeruginosa* produce ESBL. *K. pneumoniae* ATCC 700603 (positive control) and *E. coli* ATCC 25922 (negative control should be used for quality control of ESBL tests (CLSI, 2006). Enterobacteriaca, especially *Klebsiella* spp. Producing ESBLs such as SHV and TEM types have been established since the 1980s as a major cause of hospital-acquired infections. However

during the late 1990s, several community –acquired pathogens that commonly cause urinary tract infections and diarrhea have also been found to be ESBL producer. These include *E. coli, Salmonella, Shigella* and *Vibrio cholera*e (Bush and Jacoby, 2010).

2.5. Genes encodes ESBL
2.5.1. TEM

TEM-1 is the most commonly encountered beta-lactamase in Gram—negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 also responsible for ampicillin and penicillin resistance that is seen in *H. infleunzae* and *N.gonorrhoae* in increasing numbers. Although TEM-type β-lactamase are most often found in other species of Gram-negative bacteria with increasing frequency, currently 140 TEM-type enzymes have been described (Livermor, 2003).

2.5.2. SHV

SHV-1 shares 68 % of its amino acids with TEM-1 and has a similar overall structure. The SHV-1 is most commonly found in *K.pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. More than 60 SHV varieties are known. SHV-5 and SHV-12 are among three most common (Dhillon andClark, 2012).

2.5.3. CTX-M

These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e. g ceftazidime, ceftriaxone or cefpime). Rather than arising by mutation, they represent examples of

plasmid acquisition of β-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms. More than 80 CTX-M enzymes are currently known (Subha *et al*., 2001).

2.6. Detection of ESBL

ESBL testing involves two important steps. The first is a screening test with an indicator cephalosporin which looks for resistance or diminished susceptibility, thus identifying isolates likely to be harboring ESBLs. The second one tests for synergy between an oxyimino cephalosporin and clavulanate, distinguishing isolates with ESBLs from those that are resistant for other reasons (Nathisuwan *et al*., 2001).

2.6.1 Antimicrobial Susceptibility test

2.6.1.1 Dilution test

The test is performed by detecting bacterial growth in broth or agar containing antimicrobial agents in a series of two fold dilutions. The lowest concentration that inhibits the visible growth of an organism is the MIC (minimum inhibitory concentration) value. MICs provide quantitative evolution of bacterial growth inhibition by antimicrobial agents (Paterson and Bonomo, 2005).

2.6.1.2. Disk diffusion

In this method the drug concentration are created by diffusion of the testing drug through the agar from filter paper disk containing a single concentration. The size of the growth inhibition zone is used to determine

the susceptibility of the organism to the drug qualitatively (Thomson *et al.*, 2001).

2.6.2. Phenotypic confirmatory tests for ESBL production
2.6.2.1. Double –disk syngery test
In this test disks of third –generation cephalosporins and augmenting are kept 30mm apart, center to center, on inoculated Muller-Hinton agar (MHA). A clear extension of the edge of the inhibition zone of cephalosporin towards augmentin disk is interpreted as positive for ESBL production (Waksh *et al*., 2002).

2.6.2.2.Cephalosporin/clavulanate combination disks test
In this method put disk of ceftazidime and put disk which contain ceftazidime and clavulante , a ratio of cephalosporin /clavulante zone size to cephalosporin zone size of 1.5 or greater was taken to signify the presence of ESBLs activity (Khanal *et al*., 2013).

2.6.2.3. Modified Hodge test(MHT)

Is recommended by Clinical and Laboratory Standards Institute as a confirmatory test for carbapenemase production. The MHT was performed by preparing 0.5 McFarand dilution of *Escherichia coli* ATCC 25955 in 5 ml saline.Then 1:10 dilution of this preparation is streaked as a lawn on a Mueller Hinton agar plate.A10 µg meropenem disk is placed in the center of the test area. The test organism is streaked in a straight line form the edge of the plate.

Likewise *K. pneumoniae* positive and negative controls were similarly streaked. The plate was incubated ovenight at, 37˚C in ambient air for 16-24 hours positive MHT was visible in a form of a clover leaf- like shape of *E. coli* growth along the test organism streak within the diffusion zone. Negative MHT was shown by no growth of *E. coli* along the test organism streak within the diffusion zone (Sharma *et al*., 2010).

2.6.2.4. E-test
The E test ESBL strip carries two gradients: on the one end, ceftazidime; and on the opposite end, ceftazidime plus clavulanic acid. MIC is interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge (Ensor *et al.*, 2006).

2.6.2.5. Cica-Beta test

The Cica-Beta test method is a technically simple and (15min maximum) method to detect ESBLs. The method is based on the hydrolysis of a chrmogenic cephalosporin HMRZ-86, on paper strip. Four strips are available, a control strip with no inhibitor (Rupp and Paul, 2003).

2.7. Molecular diagnostic assays

2.7.1. PCR (polyermase chain reaction)

Is an in vitro technique used to replicate, or amplify, a specific region of DNA billion-fold in just a few hours. The amplification is primers directed olignucleotide primers annel to and flank the DNA region to be

amplified. PCR has rapidly become a standard method in diagnostic microbiology (Phillippon *et al*., 2002).

2.7.2. Multiplex PCR

In multiplex PCR, two or more unique DNA sequences in the same specimen are amplified simultaneously, primers used in multiplex reactions must be designed carefully to have similar annealing temperatures and to lack complementary to avoid dimerization. Multiplex PCR diagnostic assays are used in our laboratory most frequently to amplify an internal control with one set of primers and target DNA (Turner, 2005).

2.8. Activity against β-lactamase

β-lactamase inhibitors are calvulanic acid , sulbactam and tazobactam. calvulanic acid is an effective inhibitor of β-lactamase was isolated from *Streptomyces clavuligerus* in the 1970s, more than 3 decades ago. Clavulanate (the salt form of the acid in solution) showed weak antimicrobial activity alone, but when combined with amoxicillin, clavulanate significantly lowered the amoxicillin MICs against *S. aureus* , *K.pneuomniae P. mirabilis,* and *E. coli* (Bonnet, 2004).

2.8.1. Sulbactam

Is semi synthetic β-lactamase inhibitor, it is combined with certain β-lactam antibiotic to extend their activity against bacteria that are resistant to antibiotic (Avoka, 2008).

2.8.2. Tazobactam

Its action similar to sulbactam its pharmacokinetics matches with piperacillin with which has been combined for using severe infections pelvic infection, urinary and respiratory infection caused by β-lactamase producing bacilli . The spectrum of anti bacterial activity include Gram-positive and Gram -negative aerobic and anaerobic bacteria (Wright and Eliand, 2008).

2.9. Treating an ESBL infection

Possible medications used to treat ESBL infection include carbapenems , which are useful against infections caused by *E.coli* or *Klebsilla pneumoniae* bacteria fosfomycin, which is effective against ESBL bacterial infection beta- lactamace inhibitors and non beta- lactam antibiotics (Ratna *et al*., 2003).

2.10. Prevention and control

Proper infection –control practices and barriers are essential to prevent spreading and outbreaks of ESBLs -producing bacteria. The reservoir for these bacteria seems to be the gastrointestinal tract of patients. Alternative reservoirs could be the oropharynx, colonized wound and urine (Perez *et al*., 2007).

2.11. Previous study

 Mekki *et al*., (2010)**,** in Khartoum isolated hundred strains of multi drug resistant *E. coli* and *Klebsiella* spp causing nosocomial UTI from two main hospitals from Khartoum (Omdurman Teaching Hospital and Fedail

Hospital). Susceptibility testing was performed against antibiotics commonly used in treatment of UTI. *E. coli* was among the study isolates. High resistance level for third generation cephalosporin was noticed. ESBLs were detected in high prevalence among all multi drug resistant *E. coli* and *Klebsiella* species isolates (53%). Another Study carried to determine prevalence and antimicrobial susceptibly pattern of extended spectrum β-lactamases producing E. *coli*, *K. pneumoniae* and *P. mirabilis* in Khartoum. Out of 162 clinical isolates *E. coli*, *K. pneumoniae* and *P. mirabilis* represented 44.4%, 38.9% and 16.7% respectively were confirmed as ESBLs producers (Rasha and Hammad. 2016). Also Tabar *et al.,* (2016), in Iran collected 2618 urine samples, 109samples were identified as *E. coli* (4.16%). Twenty one (26.6%) of *E. coli* were ESBL positive and (73.4%) were ESBL negative. Twenty six (89.65%) of the positive samples were females and three (10.34%) were males. The average age was 32 years old and patient age ranged from 6 days to 87 years old. There was 100% susceptibility to imipenem. Twenty (68.97%) out of 29 isolates were positive for the CTX-M gene as detected by PCR

CHAPTER THREE

MATERIALS AND METHODS

3.1. Type of Study

This is a descriptive cross-sectional study.

3.2. Study area

The study was conducted in Omdurman Military Hospital. The practical part was carried out in the Research Laboratory, Sudan University of Science and Technology.

3.3. Study duration

The study was carried out during the period from April to August 2018.

3.4. Sample size

The sample size was 100 patients.

3.5. Data collection

A predesigned Questionnaire was used for collection of social

3.6. Sample collection

The specimens were collected from patients by using standard microbiological procedures. Pus from wound was collected by sterile cotton swab, urine in sterile 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 tests.

3.7. Ethical consideration

Approval has been taken from Sudan University of Science and Technology, College of Medical Laboratory and Omdurman Military Hospital.

3.8. Inclusion criteria

Patients presented with urinary tract infections, wound infections and respiratory tract infections were included in this study.

3.9. Exclusion criteria

Patients who refused to participate in this study or patients with other disease or patients who on antimicrobials within the past two weeks were excluded from this study.

3.10. Laboratory work
3.10.1. Culture

Urine specimens were cultured on CLED and blood agar media. Wound swab and sputum were cultured on MacConkey՚s agar and blood agar media, and incubated aerobically at 37˚C up to 24 hours.

3.10.2.Quality control

Used *E.coli* strains ATCC25922 for quality control of ESBL detection test

3.10.3. Purification of the isolates

The isolates were streaked on nutrient agar and incubated overnight at 37˚C. At the end of incubation period, discrete colonies were picked up and checked for purity under microscope.

3.10.4. Identification of the isolates

3.10.4. 1. Gram՚s stain
The Gram՚s stain reaction was used to help identify pathogen in specimens and culture by their Gram reaction (Gram –positive or Gram-negative) and morphology.

Gram –positive bacteria stain dark purple with crystal violet clean and not decolorized by alcohol and Gram-negative bacteria stain red because after being stained with crystal violet decolorized by alcohol. The smear was fixed by dry heat and then covered with crystal violet for 30-60 seconds the stain was rapidly washed by tap water and tipped off the slide. The stained smear was then covered with iodine 30-60 seconds. Iodine was washed off and the smear was decolorized with alcohol and immediately washed with clean water. Sfrainin was added to the smear for 30-60 seconds the red stain was then washed off with tap water and smear was subsequently air dried and examined microscopically using high resolution objective power (Cheesbrough, 2006).

3.10.5. Biochemical tests
Fermentation of glucose and lactose and production of gas and H_2S
The organism was inoculated using sterile wire loop by stabbing the butt, then blocked the pore and streaked slope media and incubated for 24 hours at 37˚C. Glucose fermentation yellow butt, lactose fermentation yellow slop, gas production in the end of the tube and H_2S production (Cheesbrough, 2006).

Citrate utilization test
In this test organism has ability to use citrate as only source of carbon. By straight wire a portion of colony was emulsified in Koser's citrate medium and incubated 24 hours at 37˚C. Positive results give blue color. Negative result shows no change (Cheesbrough, 2006).

Urease test

In this test organism produces urease enzyme breakdown urea and produce ammonia which makes the pH of medium alkaline, in the presence of phenol red indicator. The organism was inoculated in Christenin urea agar and incubated for 24 hours at 37˚C. Positive: pink color.Negative : No change (Cheesbrough, 2006).

Indole test

In this test the organism produces tryptophanase which breakdown tryptophan and produce indole, which react with kovac's reagent and give pink ring. The tested organism was inoculated into peptone water and incubated at 37˚C for overnight. The kovac's reagent was added. If there is pink ring in the medium the result was indicted as positive. If there is no pink ring in surface the result was indicated negative (Cheesbrough, 2006).

Motility test

A semi-solid agar medium was inoculated with a well-defined colony by stabbing to depth of only one third ench in the middle of the tube.The tube was incubated at 37°C for 24hrs. Motile organism will spread out into medium from the site of inoculation, while non-motile organism will remain at the site of inoculation (Cheesbrogh, 2006).

3.10.6. Antimicrobial susceptibility testing of bacterial isolates

 Bacterial isolates were subjected to antibiotic sensitivity test using the Kirby- Bauer disc diffusion method. Bacterial suspension was prepared from pure culture for each isolates. Turbidity of the suspension was adjusted to McFarland turbidity standard. Muller Hinton agar was used for disk diffusion test. The agar surface was seeded using sterile cotton swab. The plate was swabbed in three directions to insure complete distribution of the inoculums over entire plate. 15 minutes after inoculation the antimicrobial disks were applied and pressed to touch the surface of the agar using sterile forceps (Gentamicin,   Amikacin, Ciprofloxacin, Ceftazidime, Norofloxacin and Impenem) the plate was incubated at 37°C aerobically for18-24hrs. The antibiotic discs applied was evenly disturbed on the inoculated plate. For reading the zones of inhibition. Using a ruler on the underside of the plate, the diameter of each zone of inhibition was measured in mm. The end point of inhibition was where the growth started. The interpretation of the result by comparing the zone size of each antibiotic with their standard inhibition zone on chart of manufacturer.

3.10.7. Confirmatory tests for ESBLs

3.10.7.1. Modified double disc synergy   test.

Standardized inoculum of bacterial suspension equivalent to 0.5 McFarland standard turbidity of each isolate was inoculated on Muller

Hinton agar plates by using a sterile cotton swab. Then with sterile forceps the disk of the amoxicillin –clavulanate acid (30μg) was placed in the center at center of the plate and the disks of cefotaxime (30μg) and ceftazidime (30μg) were placed (center to center) at distance 20mm from amoxicillin –clavulanate acid. After incubation at 37˚C for18 hours aerobically, a clear extension of the edge of the inhibition zone of cefotaxime and ceftazidime towards amoxicillin –clavulanate acid disk was interpreted as positive for ESBL production (Mekki *et al*., 2010)

3.10.7.2. Combination disks test

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

3.11. Data 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.

CHAPTER FOUR
RESULTS

A total of 100 clinical specimens were collected from Military Hospital. The specimens were collected from 51 (51%) females and 49(49%) males, the age of participants ranges from 3 – 88 years (Table 1).

The largest number of specimens 51(51%) were collected from patients aged 31-60 years. while the fewest number of specimens 18(18%) were collected from patients aged 1-30 years (Table 2).

Cultivation of specimens on CLED agar, MaCconkey agar and Blood agar, gave bacterial growth in 65 while 35gave no growth. The isolates were identified by their colonial morphology, Grams stain and biochemical tests. The bacterial isolates were 38 organisms, from 20 wound swab and from 7 sputum. *E. coli* is the commonest organism 20(27%) among urine isolates, while *K. pneumoniae* the commenst organism 9(45%) among wound swab isolates (Table 3). The antibiotics susceptibility test was found to be all organisms suscptetible to Imipenem (100%), Ciprofloxacin (35%), Ceftaizdime, (49%) Amikacin (94%), Norofloxacin (51%) and Gentamcin (51%) (Table 4). The presence of ESBL enzymes were detected by phenotypic technique using double disc synergy and combination disc methods. The results revealed that out of 65 clinical isolates were recovered, only 12(18.5%) of isolates were ESBL positive when examined by DDST and combination disc test the

specimens were collected (Table5). The majority of ESBL producers were from urine 9 (13.8%), followed by wound swab 3(4.6%) (Table 6). *E. coli* is the most ESBLs producers (Table7). The frequency of organisms isolated from males patients compared with that isolated from females' patients was found insignificant (Table 8). The frequency of ESBLs producer in different age groups was found insignificant (p= 0.195) (Table 9).

Table 1. Distribution of clinical specimens according to the gender

Gender	Specimens			Total
	Urine	Sputum	Wound	
Females	39	4	8	51
Males	34	3	12	49
Total	73	7	20	100

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

Age groups	Specimens			Total
	Urine	Sputum	Wound swab	
1-30 years	16	0	2	18
31-60 years	35	4	12	51
61-90years	22	3	6	31
Total	73	7	20	100

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

Organism isolated	Specimens			Total
	Urine	Sputum	Wound swab	
E. coli	20	0	7	27
K. pneumoniae	13	6	9	28
P. aeruginosa	3	1	3	7
Proteus spp	2	0	1	3
Total	38	7	20	65

Table 4. Antimicrobial susceptibility patterns of the isolated bacteria

Antibiotic	*E. coli*		*K. pneomniae*		*P.aeruginosa*		*Proteus* spp	
	S	R	S	R	S	R	S	R
Gentamicin (10μg)	15 23%	12 18%	13 20%	15 23%	3 4.6%	4 6%	2 3%	1 1.5%
Norofloxacin (10μg)	10 15%	17 26%	19 29%	9 14%	2 3%	5 8%	2 3%	1 1.5%
Ciprofloxacin (1μg)	6 9%	21 32%	13 20%	15 23%	4 3%	3 8%	2 3%	1 1.5%
Ceftaizime (30μg)	7 11%	20 31%	12 18%	16 25%	4 6%	3 4.6%	2 3%	1 1.5%
Amikacin (10μg)	26 40%	1 1.5 %	26 40%	2 3%	6 9%	1 1.5%	3 4.6%	0 0%
Impenem (10μg)	27 42%	0 0%	28 43%	0 0%	7 11%	0 0%	3 4.6%	0 0%

Key: S= Sensitive
        R=Resistance

Table 5. Detection of ESBLs by confirmatory tests

Test	Positive ESBL		Negative ESBL		Total
	No.	(%)	No.	(%)	
ESBL screening	40	61,5	25	38.6	65
ESBL DDST	12	18.5	53	81.5	65
Combination test	12	18.5	53	81,5	65

Table 6. Frequency and percentage of ESBLS producers and non-ESBLS producers isolates

Type of specimens	ESBLs		Total (%)
	Positive (%)	Negative (%)	
Urine	9 (13.8%)	29(44.6%)	38(58.5%)
Wound swab	3(4.6%)	17(26.2%)	20(30.8%)
Sputum	0 (0%)	7(10.8%)	7(10.8%)
Total	12(18.5%)	53(81.5%)	65(100%)

P. value = 0.296

Table 7. Frequency and percentage of ESBLS producers and non-ESBLS producer isolates among organisms

Organism	ESBLs		Total (%)
	Positive (%)	Negative (%)	
E. coli	11(16.9%)	16(24.6%)	27(41.5%)
K. pneumoniae	1(1.5%)	27(41.5%)	28 (43.1%)
p. aeruginosa	0(0%)	7(10.8%)	7 (10.8%)
Proteus spp	0(0%)	3(4.6%)	3(4.6%)
Total	12(18.5%)	53(81.5%)	65(100%)

(P. value= 0.002)

Table 8. Frequency and percentage of ESBLS producer and non-ESBLS producers among gender

Gender	ESBLs		Total
	Positive	**Negative**	
Males	5(7.7%)	32(49.2%)	37(56.9%)
Females	7(10.8%)	21 (32.3%)	28(43.1%)
Total	12(18.5%)	53 (81.5%)	65 (100%)

P .value =0.195

Table 9. Frequency of ESBL producers among age group

age group	ESBLs		Total(%)
	Positive	**Negative**	
1-30 years	2(3.1%)	5(7.7%)	7(10.8%)
31-60 years	4(6.2%)	31(47.7%)	35(53.8%)
61-90 years	6(9.2%)	17(26.2%)	23(35.4%)
Total	12(18.5%)	53(81.5%)	65(100%)

P. value= 0.285

CHAPTER FIVE

DISCUSSON

5.1. Discussion

Increasing resistance to third generation cephalosporin has become a cause of concern especially among bacteria that cause nosocomial infections including the prevalence of ESBL among bacteria constitutes a serious threat to current β-lactam therapy leading to treatment failure (Kumar *et al.,* 2006).

Based on the results of this study out of 100 different clinical specimens examined, 65 gave bacterial growth. In this study 12(18.5%) isolates producing ESBLs. The ratio of ESBL producing bacteria was found to be (18.5%) this figure is low compared to the figure reported in a study made by Ibukun *et al*., (2001) in Nigeria who recorded ESBL production isolates (20.8%). The distribution of ESBL among *E. coli*, *K. pneumoniae, P. aeruginosa* and *Proteus* spp was 16.9%, 1.5%, 0%and 0%respectivly .This result was agreed to Omer (2013) in Sudan and Mekki *et al* (2010) reported that *E. coli* and *Klebsellia* spp are the most ESBL producers.   In this study urine was the main source of ESBL producing isolates (75%), which is in agreement with that found by Akbar *et al.,* (2007) who reported that (70.4 %) and. This fact could due to high ratio of *E .coli* among UTI patients (causing 60% of cases) than

other samples (Cheesbrough, 2006). On the other hand my results disagreed with another study conducted in India by Rudresh and Nagarathnamma (2011) where (70%) of ESBLs producing isolates were obtained from exudates.   In this study the ESBLs producers isolated from males (7.7%) and (10.8%) from females. These findings are lower than results obtained by Almugadam *et al.,* (2016) who reported ESBLs producer were 30%in males and 38% in females. The present study showed insignificant difference between ESBL and gender (P value 0.195). This result is agreement with previous study reported by Aknabi *et al.,* (2013). In this study, there was no significant difference observed in ESBL-producing isolates among age groups enrolled the study (P value 0.295). A previous study by Moyo *et al.,* (2010) reported significantly higher ESBLs production in isolates from children rather than adults.

5.2. Conclusion

The study concluded that there is high ratio of ESBLs among isolated bacteria in Military Hospital. The most organism produce of ESBLs is E. *coli*.

5.3. Recommendations

1. A larger sample size should be tested to cover a wider range of isolates

2. Advanced techniques should be used such as PCR for detection the genes and to increase the sensitivity of detection.

3. Detection of ESBL producer should be introduced as routine tests in microbiology labs for rapid detection of resistance isolates and controls their spread.

4. Prevention and contol programme of antibiotic resistance should be preformed to control the spread of ESBL producer bacteria.

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Appendices

Questionnaire

-Number of patient ( )

-Type of sample

Urine ( ) Wound swab ( ) Sputum ( )

-Sex

Female ( )   Male ( )

-Age

1-30years   ( )

31-60years ( )

61-90years ( )

-Residence

-Job

-Take antibiotic before 3months:

Yes ( )   No ( )

Figure (1) ESBL positive result of Double disk synergy test

Figure2. Negative result of Double Disk Synergy Test

Figure 3. Positive result of Combined Disk Methods

Figure 4. Negative result of Combined Disk Methods