بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Detection of Extended-spectrum β-lactamases amongst Bacteria Isolated from Patients in Atbara Teaching Hospital

الكشف عن إنزيمات البيتالاكتام واسعة الطيف لدى الباكتيريا المعزولة من المرضى بمستشفى عطبرة التعليمي

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

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الآية

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قال تعالي :

{ اللَّهُ نُورُ السَّمَاوَاتِ وَالأَرْضِ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ الْمِصْبَاحُ فِي زُجَاجَةٍ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ دُرِّيٌّ يُوقَدُ مِن شَجَرَةٍ مُّبَارَكَةٍ زَيْتُونَةٍ لّا شَرْقِيَّةٍ وَلا غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ نُّورٌ عَلَى نُورٍ يَهْدِي اللَّهُ لِنُورِهِ مَن يَشَاء وَيَضْرِبُ اللَّهُ الأَمْثَالَ لِلنَّاسِ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ }

صدق الله العظيم

(سورة النور: الآية 35)

DEDICATION

TO

My father, mother, brother and sisters.

ACKNOWLEDGEMENT

First of all, great thank to AL-MIGHTY ALLAH for offering the power to complete this work.

I would like to thank my supervisor **Prof. Humodi Ahmed Saeed,** professor of Microbiology, for his constant support and supervision during my study. I would also like to thank him for believing in my ability, for the freedom given to me to follow my instincts as well as for all the critical evaluation of my research.

ABSTRACT

Resistance to beta lactam antibiotics considered a big world health problem worldwide. The objective of this study was to detect the production of extended-spectrum beta-lactamases in clinical isolates in Atbara Teaching hospital.

A total of 100 clinical specimens were collected. These specimens were cultured on Cysteine lactose electrolyte deficient (C.L.E.D), MacConkey agar, blood agar, mannitol salt agar for primary isolate of pathogenic bacteria. Then identified depending on their colonial morphology, Gram reaction and biochemical reactions. These isolates were tested for susceptibility for several antibiotics by disc diffusion technique, seventy bacterial isolated, 50 isolates from urine specimens and 20 isolates from wound swabs, collected from both gender males and females and their age ranged from 10-70 years old. These specimens showed an isolation of 28 *E. coli, 12 K. Pneumoniae,* 20 *S. aureus* and 10 *P. aeruginosa.* The most frequent isolated organism from the urine specimens was *E. coli* with (56%=28/50), *K. Pneumoniae* (14%=7/50), *S. aureus* (30%=15/50) and *P. aeruginosa* (0%) The most common isolates in the wound swabs was the *P. aeruginosa* (50%=10/20), *K. Pneumoniae* (25%=5/20), *S. aureus* (25%=5/20) and *E. coli* (0%). In this study, imipenem was found to be the most effective antibiotic against all tested isolates where the resistant level (0%), and sensitive level (100%), then amikacin have sensitive level (92.9%) and resistant level (7.1%), gentamicin have sensitive level (57.1%) and resistant level (42.9%), ciprofloxacin have sensitive level (41.4) and resistant level (58.6%). The highest level of drug resistant by bacterial isolates was observed with cephalosporins, cefixime (94. %) ceftazidime (91.4%) and ceftriaxone (77.1%). The isolates were tested for extended-spectrum-beta–lactamases production by the Modified Double Disc Synergy test (MDDST) Result were 23(57.5%) positive to ESBL production and by CLSI Confirmatory disk diffusion method, 21(52.5%) were

positive result. The ESBL producing bacteria showed an in significant relation with the gender and specimens by two methods. This study concluded that there is a high resistance is a common phenomenon with a high percentage, must be taken in consideration by the ministry health of Sudan, and any large organization that can help in the control and prevention of β lactamases production such as the World Health organization.

ملخص الأطروحة

إن مشكلة المقاومة للمضادات الحيويه البيتالاكتام تعتبر مشكلة صحية عالمية كبيرة. وكان الهدف من هذه الدراسة الكشف عن إنتاج انزيمات البيتالاكتام واسعة الطيف في العينات السريرية من مستشفى عطبرةالتعليمي. تم جمع 100 عينة سريرية، هذه العينات تم تزريعها علي أوساط السيستين لاكتوز قليلة الشوارد، بيئة أملاح المانيتول وبيئة أجار الدم للعزل الأولي للباكتريا الممرضة. ثم تم التعرف عليها بناء علي شكل المستعمرة، وتفاعلها مع صبغة الغرام والتفاعلات البايوكيميائية. تم اختبار المعزولات التي تم الحصول عليها للحساسية للعديد من المضادات الحيوية باستخدام طريقة القرص المزدوج التأكيدية. 70 باكتريا تم عزلها، 50 باكتريا تم عزلها من البول، 20 باكتريا تم عزلها من مسحات الجروح. جمعت هذه العينات من الجنسين الذكور والإناث، وتراوحت أعمارهم بين 10 الي 70 سنة. ،حيث تم عزل 20 بكتريا من المكورات العنقوديةالذهبية، 28 باكتريا من الاشريكية القولونية، 12 باكتريا من الكلبسيلا الرئويه، 10 باكتريا من الزائفة الزنجارية. أكثر الباكتريا ترددا في عينات البول كانت الاشريكية القولونية (50/28 بنسبة 56%)، ثم الكلبسيلا الرئوية (50/7بنسبة 14%)، ثم باكتريا المكورات العنقودية الذهبية (50/15 بنسبة30 %). الزائفة الزنجارية لم يتم عزلها من مسحات البول . في مسحات الجروح الزائفة الزنجارية كانت الأكثر ترددا بنسبة (20/10بنسبة 50%)، ثم الكلبسيلا الرئوية (20/5 بنسبة 25%)، والمكورات العنقودية الذهبية (20/5بنسبة 25%) . الاشريكيه القولونية لم يتم عزلها من مسحات الجروح. وتم بعد ذلك اختبار حساسية المضادات الحيوية حيث وجد الايميبنم هوالمضاد الحيوي الأكثر فعالية بنسبة (100%) وثم الاميكاسين بنسبه حساسية (57.1%) ومقاومة بنسبة (42%)، كما تم اختيار تلاتة أنواع من السيفالوسبورينات تمت مقاومتها بنسبة عالية جدا من معظم أنواع البكتريا المعزولة، ثم تم اختيار 40 بكتريات معزوله ذات مقاومة لمعظم أنواع المضادات الحيوية ونقلت لمختبر الابحاث بجامعة السودان للعلوم والتكنولوجيا واجري عليها اختبار واسع الطيف بيتالاكتميز بطرقتين مختلفتين وأدي الي معدل انتشار بنسبه (57.5%) في الطريقة الأولي و(52.5%) في الطريقة التاكيدية. وخلصت هذه الدراسة إلى أن هناك مقاومة المضادات الحيوية هي ظاهرة شائعة بنسب عالية يجب أن تؤخذ في الاعتبار من قبل وزارة الصحة في السودان، وأي منظمة كبيرة يمكن أن تساعد في الوقاية ومنع انتاج انزيم البيتالاكتام مثل منظمة الصحة العالمية.

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1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

The current era of antimicrobial chemotherapy began in 1935 with the discovery of sulphonamides, introduction of penicillin in 1940 which ironically was discovered fortuitously in 1929 by Alexander Fleming has saved many lives, Infections that cause mortality and morbidity were treated successfully with penicillin and further by introduction of cephalosporins in the early 1960s (Kilic *et al*., 2006).

Today among the wide array of antibiotics, β-lactams are the most varied a widely used agents accounting for more than 50% of all systemic antibiotics in use, (Kumar *et al*., 2006). But after the extensive usage of penicillins and cephalosporins, bacteria have developed resistance via different mechanisms. β-lactamase enzyme production is the first developed and the most important mechanism of resistance to β-lactamses, (Kilic *et al*., 2006). β-lactamases attack the amide bond in the β-lactam ring of penicillin and cephalosporins causing disruption of molecule and convert the antibiotic to inactive penicilloic acid (Sykes, 1976). Anacyl-enzyme complex forms by deacylation, the enzyme turns in to its initial structure and binds other β-lactam antibiotics (Kilic *et al*., 2006). Disease caused by β-lactamase producing strains is refractile to therapy with β-lactam agents which are frequently used for empiric therapy leading to the serious treatment failures. In-vitro antimicrobial susceptibility shows that the isolates to be sensitive to βlactams but when agent is used for treatment, it does not work. For this reason, in-vitro determination of β-lactamase production is of clinical value in the management of patients with infectious diseases (Doern *et al*., 1987).

There are more previous studies was conducted one of them, Prevalence of AmpC β-lactamase among Gram-negative bacteria recovered from clinical specimens in Benin City, Nigeria results: Cefoxitin-cloxacillin inhibition test detected more

AmpC β-lactamase than other tests. the prevalence of Ampc β-lactamase did not differ significantly between both genders and between inpatients and out-patients (P-value>0.05). Isolates recovered from sputum had significantly higher prevalence of AmpC β-lactamase producers compared with isolates from other clinical specimens (p=0.0484). The prevalence of AmpC production was significantly higher among isolates of *Pseudomonas aeruginosa* than other isolates (p = 0.0085). Isolates that produced AmpC β-lactamase were more susceptible to the test cephalosoprins (Helen, 2016) and other study was conducted in Khartoum state, phenotypic and genotypic detection of carbapenemase enzymes producing Gram-negative bacilli isolated from patients in Khartoum state Hundred and forty-nine Gram-negative bacilli were isolated from 147 different clinical specimens. The most predominant Gram-negative bacilli isolates was *E. coli* (54.4%), followed by *klebsiella* species (29.5%). More than fifty percent of the isolates were Carbapenem resistant, fifty six percent of the resistant isolates were positive by Modified Hodge Test. By using PCR, 17.3of resistant organisms were harbored blaOXA4 gene, and 6.7% harbored blaIM gene. *E. coli* was the most bacteria that harbored the blaOXA48 followed by *Klebsiella* species. blaIMP gene was harbored only by *E. coli.* (Dahab *et al., 2017*).

1.2. Rationale

The antibiotic resistance is a world-wide health problem, Infections caused by ESBL-positive bacteria results in high patient morbidity and mortality making their detection clinically important as they cannot be detected in routine susceptibility testing. This study aims to detect the extended-spectrum β-lactamases amongst bacteria isolated from patients in Atbara Teaching Hospital, to determine the efficacy of used antibiotics in treating different bacterial infections.

1.3. Objectives

1.3.1. General objectives

To investigate extended spectrum beta-lactamases from different clinical isolates.

1.3.2. Specific objectives

a) To isolate bacteria from different clinical specimens.
b) To perform antimicrobial susceptibility testing by using Kirby -disk diffusion method.
c) To detect ESBLS amongst bacterial isolates.
d) To determine the frequency of β-lactamases amongst bacterial isolates.

2. LITERATURE REVIEW

2. 1. Discovery of beta-lactam antibiotics

Globally bacterial infectious diseases have been responsible for a very large number of deaths and mankind has been in a constant state of conflict with bacteria since time immemorial for the diseases they produce.

Antibiotic was discovered in 1928 when Alexander Fleming published his famous discovery of Staphylococcal colonies being inhibited by a contaminating Penicillium mould, Upon subsequent experiments on selected bacterial cultures, he noted that the extract of fungal culture possessed "inhibitory, bactericidal and bacteriolytic properties "against most gram positive bacteria such *Staphylococcus aureus, Bacillus anthracis* ,*Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Corynebacterium diphtheria* but not against Gram negative bacteria such *Escherichia coli, Klebsiella pneumoniae*, *Vibrio cholerae*, and *Hemophilus influenza*, he coined the termed "penicillin" to refer to the "mould broth filtrate" that had antibacterial properties, since it was derive from Penicillium spps (Fleming,1929). Although Fleming's discovery was a matter of providence, investigation into the anti-bacterial properties of fungi had begun much earlier. The observation that fungus can inhibit bacteria was first document by a French physician, Ernest Duchesne in 1897. In his dissertation, he described the antibacterial properties of the fungus Penicillium glaucum (Shampo *et al*., 2000). (Duckett, 1999).

Fleming did not undertake any experiments to evaluate the clinical utility his discovery, but this work was continued by Howard W. Florey and Ernst Boris Chain in 1940 who successfully demonstrated the utility of purified penicillin by conducting in vivo studies using mice deliberately infected

with Streptococcus (Florey, 1942). This was followed by successful clinical trials in 1941 and the drug was then made available for therapeutic use (Abraham, 1947).

At the same time other biologically derived compounds with antibacterial property were being discovered from *actinomycetes*. In 1940, Selman A. Waksman, along with his students, discovered Streptomycin from *Streptomyces griseus* (Waksman, 1940). In order to differentiate such biologically derived compounds from other chemotherapeutic agents, he coined the term "antibiotic" (Waksman, 1947).

These initial discoveries led to vigorous interest in searching for other Antibiotics and one such attempt were made by Giuseppe Brotzu in 1948, which discovered antibacterial activity in crude extracts of the mould Cephalosporium acremonium (now known as Acremonium chrysogenum). Of the three compounds characterized in the culture extract, Cephalosporin C possessed most useful antibacterial property (Crawford *et al*., 1952).

2.2. Classification of beta-lactam antibiotics

Beta-lactam antibiotics include the following five classes (Yao *et al*., 2007).

a) Penicillins e.g., ampicillin, oxacillin.
b) Cephalosporins e.g., cephalexin, cefaclor.
c) Penems e.g., imipenem, meropenem.
d) Carbacephems e.g., loracarbef.
e) Monobactams e.g., aztreonam.

2.3. Mechanism of action of beta-lactam antibiotics

During the course of bacterial growth, the cell wall is continuously remodeled as the old ones are broken down and new ones are formed. transglycosylase, transpeptidase, carboxypeptidase and endopeptidase are the bacterial enzymes that are involved in remodeling and synthesis of cell walls. These are a family of acyl serine transferases that catalyze the polymerization (transglycosylation) and cross-linking (transpeptidation) of the glycan strands of peptideglycan. D-alanyl-D-alanine, a natural substrate of these enzymes is structurally similar to beta-lactam antibiotic (Pitton, 1972).

This similarity tricks the bacterial enzymes into binding with beta-lactam antibiotics in their active sites. It is for this reason that the transpeptidase enzymes are also known as Penicillin Binding Proteins (PBPs). By blocking the active site of these enzymes, beta lactam antibiotics deprive them of their natural substrates. This prevents the cross linking of cell wall and ultimately the cell dies dues to osmotic instability (Pitton, 1972). Bacteria evolved quickly to counter the beta-lactam antibiotics and resistance appeared in *S.aureus* within a year of penicillin's use (Rammelkam *et al*., 1942).

2.4. Resistance to beta-lactam antibiotics

Resistance to beta-lactam antibiotics can be intrinsic or acquired; most Gram-Negative bacteria are intrinsically resistant to penicillin. Bacteria have evolved to counter the adverse effects of beta-lactam antibiotics in the following three diverse ways (Abraham, 1940).

a) Mutations leading to loss or under-expression of porins that disallow entry of beta-lactams.

b) Production of new penicillin binding proteins that have low affinity to beta-lactams, expulsion of beta-lactams from periplasmic space mediated by efflux pumps.

c) Production of enzymes that hydrolyze beta-lactam rings.

Of all these methods, the enzymatic inactivation by beta-lactamases is the most common strategy adopted by the bacteria. The first evidence of enzymatic inactivation of penicillin came in 1940, even before the antibiotic was used in therapeutics. Abraham and Chain were able to demonstrate an enzyme in *E. coli* that hydrolyzed penicillin; they named it "penicillinase", Beta-lactamase is a broader name given to bacterial enzymes that hydrolyze various beta-lactam antibiotics. In case of Gram-positive bacteria, these enzymes are excreted outside the cell whereas in Gram negative bacteria, they are present in the periplasmic space (Abraham, 1940).

2.5. Beta-lactamases

Beta-lactamases are classified as serine beta-lactamase when they have a serine radical or as metallo-beta-lactamases (MBLs) when they have zicion at the enzyme's active site. Typically, the inactivation of beta-lactam antibiotic involves acylation and deacylation steps. In the acylation step, the beta-lactam ring is opened forming an enzyme-acyl complex, which is then deacylated from serine following hydrolysis. While the acylation step requires nucleophilic serine, deacylation requires hydrolytic water molecule (Livermore, 1995).

2.5.1. Classification of beta lactamases

"Molecular" classification based on the amino-acid sequences of the beta-lactamases. In his classification, he divided beta-lactamases into two groups: Class A (serine betalactamases) and Class B (metallo-beta-lactamases) (Ambler, 1980) Class C, consisting of AmpC beta-lactamases was added subsequently by Jaurin and Grundstrom in 1981 (Jaurin, 1981). In 1988, Huainan Pet alexpanded this classification by including Class D, which encompasses oxacillinases (OXA-type) (Huovinen *et al*., 1988).

2.5.2. Different Types of beta lactamases

2.5.2.1 Extended Spectrum Beta Lactamases (ESBLs)

Extended spectrum Beta-lactamases (ESBLs) are mutant with a wide range of activity than for their parent molecules. They hydrolyze third and fourth generation cephalosporin's and aztreonam but do not affect the second-Generation cephalosporin's and remains susceptible to beta lactamases inhibitor. The most common plasmid–mediated beta- lactamases in Enterobacteriaceae are TEM-1,

TEM-2 and SHV1.Classical ESBLs are derived from TEM and SHV whereas non-classical ESBLs are derived from enzyme other than TEMand SHV. Classical ESBLs are primarily found in *E. coli* and *Klebsiellae* species. They differ from their parent enzyme only by 1-4 amino acids non-classical ESBLs are less common than classical ESBLs .it includes CTX-M and OXA (Jacoby and Munoz, 2005).

2.5.2.2. Ampcs

They are not inhibited by β-lactamase inhibitors. That are repressed and produced at low levels. Plasmid mediated AmpCs are also inducible. Two mechanisms responsible for AmpCs activity in *E. coli* are mutation in AmpCs promoter and attenuator regions resulting in AmpCs over expression and acquisition of plasmid-carried AmpCs genes (Philippon *et al*., 2002).

2.5.2.3. Sulfgydryl (SHV)

The most prominent beta-lactamases produced by Enterobacteriacea are the sulfhydryl (SHV) family, the first reported SHV had narrow spectrum of activity Derivatives of SHV-1have been evolved due to the accumulation of point mutation at the active site of enzyme. these derivatives have an extended spectrum of activity which is capable of inactivating third –generation cephalosporin (Heritage *et al*.,1999).

2.5.2.4. Plasmid Encoded Transposable Element beta lactamases (TEM-1)

It is one of the most well known in producing antibiotic resistance. It confers resistance to penicillin and early cephalosporin. Almost 90% of resistant *E.coli* is due to the production of TEM-1. By opening the active site to β-lactam substrates enhances the susceptibility of enzyme to β-lactamase inhibitors, 140 TEM-type enzymes have been described. TEM-10, TEM-12, TEM-1 and TEM-are most common (Bradford, 2001).

2.5.2.5. Oxacillinase (OXA)

The OXA-type (oxacillinhydrolyzing) enzymes are produced by Enterobacteriaceae and *P. aeruginosa* pose resistanse against amino and ureido penicillin and high-level hydrolytic activity against cloxacillin, oxacillin and methicillin. clavulanic acid strongly inhibits the activity of this enzyme they belong to ambler class D and thus possess and active serine site as class A and C beta–lactamases (Naas and Nordmamnn 1999).

2.5.3. β-lactamases inhibitors

Clavulanic acid, sulbactam, tazobactam these agents are potent inhibitors of many kind of β-lactamase and are used together with broad spectrum penicillins to protect them from inactivation (Bradford, 2001).

2.6. Tests for detection of β-lactamases

2.6.1. Acidimetric method

Hydrolysis of the β-lactam ring generates a carboxyl group, acidifying un-buffered systems. The resulting acidity can be tested in tubes or on filter papers (David and Brown, 2005).

2.6.2. Iodometric method

Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolorizing starch-iodine complex. This reaction can be exploited to detect β-lactamase activity in tubes or on paper strips (Bidya and Suman, 2014).

2.6.3. Nitrocefin test

Is a chromogenic cephalosporin that changes from yellow to red on hydrolysis? It provides the most sensitive test for most β-lactamases, exceptions being staphylococcal penicillinase and ROB-1, an uncommon plasmid-mediated enzyme of haemophili. A 0.5 ml nitrocefin solution is prepared by dissolving 2.58 mg of powder in 0.5 mL of dimethylsulphoxide (DMSO) then diluting with 9.5 mL of 0.1 M phosphate buffer, pH 7.0. This solution is stable for 10 days at 4°C in a foil-wrapped bottle. Glasscontainers should be used, since DMSO degrades plastics. Colonies of the test isolates are scraped from nutrient agar plates and are suspended in 20 µL volumes of 0.1 M phosphate buffer pH 7.0, to produce a dense suspension on a glass slide, and 20 µL amounts of the nitrocefin solution are added. β-Lactamase activity is indicated by a red colorwithin 1-2 minute (David and Brown, 2005).

2.6.4. Microbiological tests of β-lactamase activity

β-Lactamase activity can be detected biologically by demonstrating the loss of activity of a β-lactam agent against a susceptible indicator organism. There are several variations, including the cloverleaf (Hodge) method, which is highly sensitive for staphylococci, and the Masuda Double Disc Method, which can be used with whole cells or cell extracts of test strain (David and Brown, 2005).

2.6.5. 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 McFarland dilution of *E. coli* ATCC 25922 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 from the edge of the disk to the edge of the plate. Likewise, *K. pneumoniae*

positive and negative controls were similarly streaked. The plate was incubated overnight at 35±2°C in ambient air for 16–24 hours. After incubation, 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 (Basher, 2016).

2.6.7. Double disk synergy test (DDST)

The double disk synergy test using β-lactam and β-lactamase-inhibitor disks is a convenient method of detecting extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacilli (Basher, 2016). Standardized inoculums of bacterial suspension equivalent to 0.5 McFarland standard turbidity of each is inoculated on Mueller-Hinton agar plates by using a sterile cotton isolate swab, then with sterile forceps the disk of amoxicillin- clavulanic acid (MAC 30ug) is placed at centre of plate and the disks of cefotaxime (30ug) and ceftazidime (30µg) are placed (centre to centre) at distance 20 mm from MAC 30ug disk. After incubation at 37 ºC for 18hours aerobically, a clear extension of the edge of the inhibition zone of cephalosporin towards MAC 30ug disk was interpreted as positive for ESBL production (Almugadam *et al*., 2016).

2.6.8. EDTA-synergy test

EDTA inhibition of β-lactamase activity is used to differentiate a metallo β-lactam as from other β-lactamases. To perform EDTA-disk synergy test an overnight culture of the test isolate is suspended in a McFarland broth (turbidity No. 0.5) and swabbed on a Mueller-Hinton agar plate. After drying, 10-µg imipenem disks together with a blank filter paper disk are placed on Mueller–Hinton agar plate 10 mm apart. 10 µL of 0.5 M EDTA solution was applied to the blank disk.

(i.e. approximately 1.5 mg/disk). After overnight incubation, the presence of an enlarged inhibition zone of imipenem indicates a positive EDTA-synergy test (Basher, 2016).

2.6.9. E Test

Also available and the manufacturer suggests that these are suitable for detecting E test with a ceftazidime or cefotaxime gradient at one end and a ceftazidime or cefotaxime plus clavulanate gradient at the other can be used to detect ESBLs. They should be used in accordance with the manufacturer's package insert, which specifies a heavier inoculum than for the BSAC disc test method. ESBL production is inferred if the ratio of the MIC of cephalosporin alone to the MIC of cephalosporin plus clavulanate is eight or more. E test containing a cefepime/ clavulanate gradient are all ESBLs, though there is only limited supportive data, particularly for strains with very weak ESBLs, such as TEM-12 (David and Brown 2005).

2.6.10. Combined disc methods

These depend on comparing the zones given by discs 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 States Clinical Laboratory Standards Institute (CLSI; formerly NCCLS) recommends comparison of the zones given by cefotaxime 30 µg versus cefotaxime + clavulanate 30 + 10 µg and ceftazidime 30 µg versus ceftazidime+clavulanate 30+10µg discs (David *et al*., 2005).

3. MATERIALS AND METHODS

3.1. Type of Study

This is descriptive across sectional study.

3.2. Study area

The study was conducted in Atbara Teaching Hospital, Atbara it located in River Nile state in northeastern Sudan.

3.3. Study duration

From March to September 2018.

3.4. Study population

The study was done on 100 patients with different bacterial infections, includes both male and female, from ages 10-70 years.

3.5. Sample Size

A total of 100 samples were enrolled in this study.

3.6. Collection of Specimens

Different types of specimens were collected, include: urine and wound swabs.

3.7. Isolation and Identification

Specimens were inoculated on different types of culture media including Cysteine lactose electrolyte deficient (CLED), MacConkey agar, Blood agar, then the cultured plates were incubated at 37°C for 24 hours, cultures were examined macroscopically for colonial morphology, and Gram stain was performed from suspected colonies. All gram-positive cocci are subcultured on mannitol salt agar and DNA media medium for purity of *S. aureus*, and all isolates subcultured on MacConkey agar media for purity and further identification tests, incubated at 37°overnight. different standard biochemical reactions, including oxidase, Kligler Iron Agar (KIA), urease production, citrate utilization, indole production, and motility tests were performed for gram negative bacteria to provide full report about the isolated microorganism. (All media mentioned above were obtained from microbiology Lab of Sudan University of science and Technology).

3.8. Antimicrobial susceptibility test (Kirby-Bauer disk diffusion method)

Antimicrobial susceptibility test was carried out by Kirby-Bauer disc diffusion method (Wayne, 2008) this test, Standardized inoculums of bacterial suspension equivalent to 0.5 McFarland standard turbidity of each isolate, Mueller- Hinton agar plates were prepared, Sterile cotton swabs were dipped in the culture broth and then soaked swabs were rotated against the upper inside wall of the tube to remove excess fluid, The entire agar surface of the plate was streaked with the swab three times, turning plate at 60 degree angle between each streaking, The medium was allowed to dry for 60 second, using antibiotic disc dispense following discs were released on to the surface, the antimicrobial discs used were Amikacin AK30µg, Imipenem IPM10µg, Gentamicin GEN10µg, ciprofloxacin CIP5µg, cefixime CFM10µg, Ceftazidime CAZ30µg, Ceftriaxone CTR30µg. The discs were pressed down with sterile bacteriological loop to secure. The plates were incubated at 37°C and examined after 24 hours. The zone diameters were interpreted as per Clinical Laboratory Standards Institute recommendations, quality control was performed to measure the effectiveness of antimicrobial agents by using a control *Escherichia coli* ATCC 25922, obtained from the central public health laboratory.

3.9. Detection of ESBL Production

3.9.1. Modified Double Disc Synergy test (MDDST)

All the isolates which showed a diameter of resistance for 3rd generation cephalosporins in the antimicrobial susceptibility testing were selected for checking for ESBL production, this test was done by using a disc of amoxicillin-clavulanate (20/10µg) along with cephalosporins; 3rdgeneration: cefotaxime, ceftriaxone, ceftazidime and 4thgeneration, cefepime. a lawn culture of the organisms was made on a Mueller-Hinton agar plate, an amoxicillin-clavulanate disc (20/10µg) was

placed in the centre of the plate and the discs of 3rd and 4thgeneration cephalosporins were placed 15mm and 20mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc. Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for ESBL production (Kaur *et al*., 2013).

3.9.2. Confirmatory Test for detection of ESBLs

A lawn culture of the organisms was streaked on a Mueller-Hinton agar plate, then cefotaxime and ceftazidime discs, alone and in combination with clavulanic acid were placed on the surface of the agar plate. A ≥ 5-mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs its zone when tested alone indicated positive ESBL production (Nadeem *et al*., 2009).

3.10. Data analysis

Data obtained were analyzed using descriptive statistics performed using SPSS version 16.0, to check the statistical significance and Excel 2007. The p-value that considered significant was < 0.05.

3.11. Ethical consideration

This study was approved by the college of Medical Laboratory Science, Ethical committee, college of Medical Laboratory Science department of microbiology supervision, Sudan University of Science and Technology and from Atbara Teaching Hospital and patients, specimens we are collected from them.

RESULTS

One hundred different clinical specimens were collected from patients admitted to Atbara Teaching Hospital during the period from March to September 2018.

The specimens were collected, include urine and wound swabs from both gender males and females and their age ranged from 10-70 years table (1) and table (2). Specimens were processed on different types of culture media including Cysteine electrolyte deficient (CLED), MacConkey agar, Blood agar, then the cultured plates were incubated at 37°C for 24 hours, cultures were examined macroscopically for colonial morphology, and Gram stain was performed from suspected colonies. All gram-positive cocci are subcultured on mannitol salt agar and DNA media medium for purity of *S. aureus*, and all isolates subcultured on MacConkey agar media for purity and further identification tests, incubated at 37°C overnight. different standard biochemical reactions, including oxidase, Kligler Iron agar (KIA), urease production, citrate utilization, indole production, and motility tests were performed for gram negative bacteria to provide full report about the isolated microorganism. Seventy bacterial isolates, (50) bacteria isolated from urine specimens and (20) isolates from wound swabs, as shown in table (3). These specimens showed an isolation of 28 *E. coli, 20 S. aureus,* 12 *K. pneumoniae, and 10 P. aeruginosa.* 28 isolates of *E. coli* were obtained from the urine specimens as the most frequent isolated organism. 7 isolates of *K. Pneumonia*e were obtained from urine specimens and 5 from wound swabs.15 isolates of *S. aureus* were obtained from urine specimens, 5 from wound swabs. 10 isolates of *P. aeruginosa* as the most frequent isolates from wound as shown in table (4). The antimicrobial discs used were Amikacin AK30µg, Imipenem IPM10µg, Gentamicin GEN10µg, ciprofloxacin CIP5µg, cefixime CFM10µg, Ceftazidime CAZ30µg, Ceftriaxone CTR30µg. In this study, Imipenem was found to be the most effective antibiotic against all the tested isolates where the resistant level (0%), and sensitive level (100%), then amikacin have sensitive level (92.9%) and resistant level (7.1%), Gentamicin have sensitive level (57.1%) and resistant level (42.9%), Ciprofloxacin have sensitive level (41.4) and resistant level (58.6%). The highest level of drug resistant was observed among

cephalosporins: cefixime (94%) ceftazidime (91.4%) and ceftriaxone (77.1%) by the most of bacterial isolates, as shown in table (6). The isolates were tested for extended-spectrum-beta–lactamases production by the Modified Double Disc Synergy test (MDDST) Result was 23(57.5%) positive to ESBL production and confirmed by CLSI Confirmatory disk diffusion method, 21(52.5%) were positive result. as shown in table (7). The ESBLS producing bacteria showed an in significant relation with the gender and specimens by two results shown in tables (8-11).

Table 1. Frequency of different clinical specimens

Specimens	**NO**	**%**
Urine	50	(71.4%)

Wound	20	(28.6%)
Total	70	(100%)

Table 2. Distribution of enrolled patient according to the gender

Gender	**NO**	**%**
Male	30	(42.9%)
Female	40	(57.1%)
Total	70	(100%)

Table 3. Distribution of clinical specimens according to the gender

Specimens	**Male**	**Female**	**Total**
Urine	**14**	**36**	**50**
Wound swabs	**16**	**4**	**20**
Total	**30**	**40**	**70**

Table 4. Distribution of bacterial isolates according to specimens

Isolates	**Urine**	**Wound swabs**	**Total**
E. coli	28	0	28

S. aureus	15	5	20
K. pneumoniae	7	5	12
p. aeruginosa	0	10	10
Total	50	20	70

Table 5. Distribution of clinical specimens according to the age groups

Specimens	**(10-25)**	**(26-40)**	**(41-55)**	**(56-70)**	**Total**
Urine	**5**	**30**	**10**	**5**	**50**
Wound swabs	**2**	**2**	**6**	**10**	**20**
Total	**7**	**32**	**16**	**15**	**70**

Table 6. Susceptibility to the antibiotics discs among different clinical isolates

	AK (10)		IPM (10)		GEN (10)		CIP (5)		CFM (0)		CAZ(30)		CTR30)	
Isolates	S	R	S	R	S	R	S	R	S	R	S	R	S	R

S. aureus	20	0	20	0	13	7	13	7	4	16	6	14	12	8
E. coli	28	0	28	0	15	13	6	22	0	28	0	28	0	28
K.pneumoniae	12	0	12	0	8	4	6	6	0	12	0	12	4	8
p. aeruginosa	5	5	10	0	4	6	4	6	0	10	0	10	0	10
Total %	92.9	7.1	100	0	57.1	42.9	41.4	58.6	5.7	94	8.6	91.4	22.9	77.1

Key: S: sensitive, R: resistance, AK (10): Amikacin, IPM (10): Imipenem, GEN (10): Gentamicin, CIP (5): Ciprofloxacin CFM (10): Cefixime, CAZ (30): Ceftazidime, CTR (30): ceftriaxone.

Table 7. Detection of ESBLS according to techniques

Isolates	MDDST		CLSI Confirmatory disk diffution method		Total number of isolates
	Positive	Negative	Positive	Negative	
E. coli	12	11	9	14	23
K. pneumoniae	5	3	6	2	8
p. aeruginosa	6	3	6	3	9
Total %	23(57.5%)	17(42.5%)	21 (52.5%)	19(47.5%)	40(100%)

Table 8. ESBL producing isolates by the Modified Double Disc Synergy test (MDDST) in relation to gender

Gender	No. tested	No. positive for ESBLs%	P-value
Male	10	8(20) %	.096
Female	30	15(37.5)	

Table 9. ESBL producing isolates by the Modified Double Disc Synergy test (MDDST) in relation to specimens

Specimens	No. tested	No. positive for ESBLs%	P-value
Urine	26	15(37.5%)	.524
Wound	14	8(20%)	

Table 10. ESBL producing isolates by the Confirmatory disk diffusion method in relation to gender

Gender	No. tested	No. positive for ESBLs%	P-value
Male	10	7(17.5%)	.181
Female	30	14(35%)	

Table 11. ESBL producing isolates by the Confirmatory disk diffusion method in relation to specimens

Specimens	No. tested	No. positive for ESBLs%	P-value
Urine	26	14(35%)	.554
Wound	14	7(17.5%)	

5. DISCUSSION

5.1. Discussion

The infections that are caused by multidrug-resistant gram-negative bacilli that produce various beta-lactamases enzymes have been reported with an increasing frequency and they are associated with a significant morbidity and mortality. (Deshmukh *et al*., 2011). This study detected the presence of ESBL mediated resistance in different clinical isolated of patients in Atbara Teaching Hospital. the antimicrobial susceptibility testing was done to all isolates, Imipenem is the mostly effective antibiotic against all the tested isolates where the resistant level (0%), and sensitive level (100%), this result is agreement with study of (Jones et al., 2002), (Iroha *et al*., 2015) and (Turner, 2009). The highest level of drug resistant was observed among cephalosporins: cefixime 94%, ceftazidime 91.4% and ceftriaxone 77.1%, by mostly of bacterial isolates, this results agreement with study of (Devaraju *et al*., 2016). The prevalence of ESBL-producing isolates was found to be 23 (57.5%) positive result by the Modified Double Disc Synergy test (MDDST) and confirmed by CLSI Confirmatory disk diffusion method, 21(52.5%) were given positive result. this study showed insignificant difference in the distribution of ESBL production in relation to gender of patients and source of specimens, agreement with the findings of (Yusuf *et al*., 2013). All our isolates that were found to be positive ESBL producers by the MDDST 23 (57.5%) also showed positive results in disk diffusion 21 (52.5%) method except 2 isolates which were positive for ESBL by MDDST but showed negative results with the disk diffusion method because they showed synergy with cefepime only in MDDST but not with any of the other third-generation cephalosporins which were used. this results are similar, to some extent, to those reported by Kaur *et al*. as they found that of the 136 isolates, 112 (82%) and 102 (75%) were positive for ESBL by the MDDST and the disk diffusion methods respectively. Ten (7.4%) isolates (eight *E. coli* and two K. *pneumoniae*) were positive for ESBL by MDDST, yielded negative results with the disk diffusion method and therefore they reported that cefepime improves the sensitivity of

MDDST in detecting ESBL producing bacteria (Kaur et al., 2013). According to the mentioned studies, it seems that the prevalence of β-lactamases-producing Enterobacteriaceae in different parts of the world can be varied from 0% to more than 70%. This difference could be due to the factors such as differences in the type and mode of antibiotic consumption that cause genetic mutations in bacteria and producing the mentioned enzymes. (Manch *et al.*, 2006).

5.2. conclusion

This study concluded that there a highly drug resistance is a common phenomenon with a high percentage, must be taken in consideration by the ministry health of

Sudan, and any large organization that can help in the control and prevention of beta lactamases production such as the World Health organization. This study showed a high prevalence of ESBLs (57.5%) by the Modified Double Disc Synergy test (MDDST) and (52.5%) by CLSI confirmatory disc diffusion method, the prevalence of ESBLs producing *E. coli* was higher than other isolates.

5.3. Recommendations

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

2. Detection of beta- lactamase producers should be introduced as routine tests in microbiology labs for rapid detection of resistant isolates and to control their spread, especially for newly admitted patients to the hospitals.
3. Control the type and mode of antibiotic consumption because the miss used that cause genetic mutations in bacteria and producing the mentioned enzymes.
4. Genetic detection to determine the responsible genes for ESBLS production.

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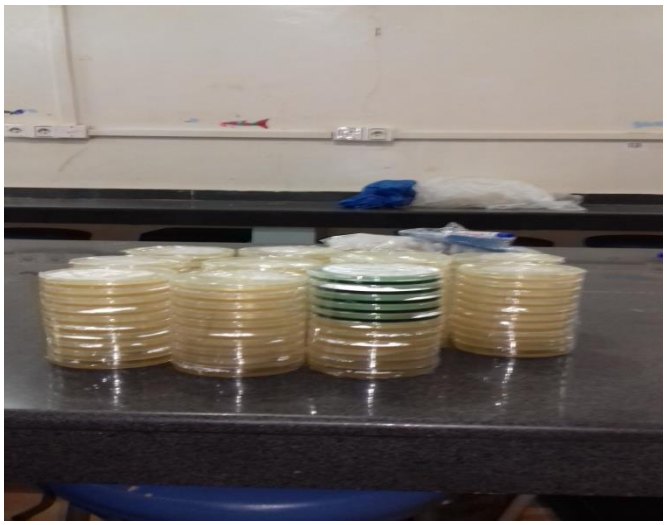
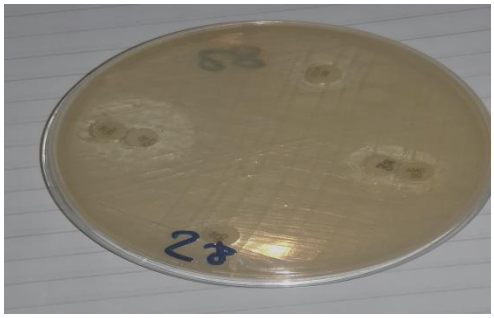
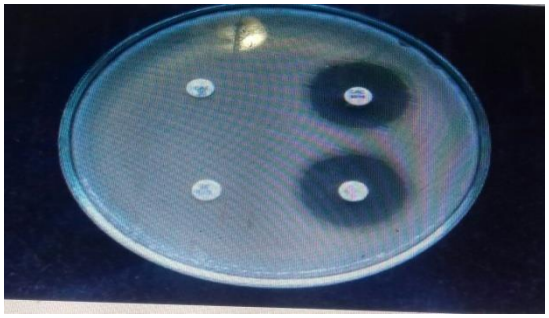
Appendices

Culture media



Positive result of modified Double Disk Synergy Test

Positive result of Combined Disk Methods