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Sudan University of Science and Technology

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Detection of Extended-Spectrum β-lactamase and Amp-C β-lactamase among Bacteria Isolated from Patients in Some Hospitals in Khartoum State

الكشف عن انزيمات البيتالاكتام واسعة الطيف وانزيم أمبلار(ج)في البكتيريا المعزولة من المرضى في بعض المستشفيات في ولاية الخرطوم

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DEDICATION

To my mother,

Father, brothers, sisters,

and friends

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First of all thanks to ALMIGHTY ALLAH for helping research and giving me the strength to complete this dissertation.

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ABSTRACT

Production of β-lactamase enzymes by Gram-negative bacteria is the most common mechanism to acquire drug resistance to β-lactam antibiotics. Limitations in detecting extended spectrum β-lactamases (ESBL) and Amp-C β-lactamases have contributed to the uncontrolled spread of bacterial resistance and are of significant clinical concern.

This study was conducted to detect extended spectrum β-lactamase (ESBL) and Amp-C β-lactamase among pathogenic bacteria.

A Total of 100 clinical specimens (74 urine and 26 wound swab) were collected from patients in Omdurman Teaching Hospital, Asia Hospital and Omer Sawy Military Hospital. These specimens were collected from both males and females. The age of patients was ranged from 10-95 years. The specimens were cultured on MacConky agar, Blood agar and cysteine lactose electrolyte deficiency (CLED) agar. The isolates were identified by colonial morphology, Gram stain and biochemical tests. Isolated bacteria were subjected to antimicrobial sensitivity test. The phenotypic test double disc synergy was used to detect ESBLs, while the disc approximate test used to detect Amp-C enzyme.

Fifty-eight (58%) of specimens gave bacterial growth, while (42%) specimens showed no bacterial growth. 58 of bacteria isolated were identified. Urine was the major source of the bacterial isolates 42(72.41%), comprising of the wound swabs 16(27.59%). The most common frequent isolates were *Klebsiella pneumoniae* 26 (44.8%), followed by *Escherichia coli* 14(24.1%), *Proteus spp* 11(19%), *Pseudomonas aeruginosa* 6(10.3%) and *Citrobacter spp*1(1.8%). The

result of sensitivity test revealed that 45 (77.6%) of isolated were resistant to ceftazidime. Only 10(17.2%) of the isolates were ESBLs positive. These were 5(8,6) *Klebsiella pneumoniae,* 3(5,2%) *Escherichia coli* and 2(3.4%) *Proteus spp*. Only 4 (6.9%) of isolates were Amp-C β-lactamase positive. These were 2(3.4%) *Pseudomonas aeruginosa*, 1(1.8%) *Klebsiella pneumoniae* and 1(1.8%) *Proteus spp*.

The association between ESBL producer organisms and gender was found statistically insignificant (*p*.value = 0.635), while the association between Amp-C β-lactamase producers and gender was found statistically significant (*p*. value = 0.03). The association between ESBL producer organisms and age groups was found statistically insignificant (*p*. value = 0.09), while the association between Amp-C β-lactamase producer and age groups was found statistically significant (*p*. value = 0.04).

This study concluded that there is high ratio of ESBLs producers in hospitals and the most producer was *Klebsiella pneumoniae*.

Advanced technique such as PCR test for detection of ESBL and Amp-C β-lactamase is required to confirm the results of this study. Use of antibiotic should be controlled in hospitals and prevention of disease by measures other than the use of antibiotics to reduce antibiotic resistance.

المستخلص

يعد إنتاج إنزيمات البيتالاكتاميزبواسطة البكتيريا سالبة الجرام الآلية الأكثر شيوعًا لإ كتساب مقاومة لأدوية المضادات الحيوية البيتالاكتام. وقد ساهم الإكتشاف المحدود لإنزيمات البيتالاكتاميز واسعة الطيف وإنزيم أمبلار (ج) في الانتشار غير المنضبط للمقاومة البكتيرية وهي ذات أهمية سريرية كبيرة.أجريت هذه الدراسة للكشف عن إنزيم البيتالاكتاميز واسع الطيف وإنزيم أمبلار (ج) بين البكتيريا المسببة للأمراض. جمعت مائة عينة (74 عينة بول و 26 عينة جروح) من المرضى في مستشفى أم درمان التعليمي ، ومستشفى آسيا ومستشفى عمرساوي العسكري .وقد جمعت هذه العينات من كل من الذكور والإناث ، وتراوحت أعمار المرضى بين 10-95 سنة. زرعت جميع العينات في الوسط الزراعي المناسب لها، واستخدم شكل المستعمرة، صبغة الغرام والإختبارات البيوكيميائة لمعرفة هوية هذه العينات. كشف عن نمط استجابة البكتيريا لبعض الأدوية. تم استخدام اختبار الأقراص المزدوجة للكشف عن إنزيم البيتالاكتاميز واسع الطيف، في حين استخدم الاختبار التقريبي للقرص للكشف عن إنزيم أمبلار(ج) .

النتيجة أظهرت أن(58%) من العينات نمت و(42) لم تنمو.وتم التعرف على 58 من البكتيريا المعزولة. وكان البول هو المصدر الرئيسي للعزلات البكتيرية التي تم جمعها 42 (72.4%)، بما في ذلك عينة الجروح16(27.59%) .أكثر أنواع البكتيريا التي تم عزلها هي الكليبسيلة الرئوية 26 (44.8%) تتبعها العصيات القولونية 14(24.1%)، البكتيريا المتقلبة 11(19%)، الزائفة الزنجارية 6(10.3%) و البكتيريا اليمونية 1(1.8%). كشفت نتيجة اختبار الحساسية أن45 (77.6%) من الباكتيريا المعزولة كانت مقاومة للسيفالوسبورين (سيفتازيديم).وجد أن 10(17.2) من البكتيريا المعزولة منتجة لإنزيم البيتالاكتاميز واسع الطيف و هذه كانت 5(8.6%) الكليبسيلة الرئوية، 3(5.2%) العصيات القولونية و 2(3.4%) البكتيريا المتقلبة. ووجد أن أربعة فقط (6.9%) من البكتيريا المعزولة منتجة لإنزيم أمبلار(ج) وهذه كانت 2(3.4%) الزائفة الزنجارية، 1(1.8%) الكليبسيلة الرئوية و 1(1.8%) المتقلبة.

وجد إحصائيا: أنه لايوجد علاقة بين البكتيريا المنتجة لإنزيم البيتالاكتاميز الواسع الطيف وجنس المريض وكانت القيمة الأحتمالية تساوي(0.635)، بينما وجدت علاقة بين البكتيريا

المنتجة لإنزيم أمبلار (ج) وجنس المريض وكانت القيمة الإحتمالية تساوي(0.03). ولم توجد علاقة بين البكتيريا المنتجة لإنزيم البيتالاكتاميز واسع الطيف والفئات العمرية حيث كانت القيمة الإحتمالية تساوي(0.09). بينما وجدت علاقة بين البكتيريا المنتجة لإنزيم الأمبلار (ج) , والفئات العمرية وكانت القيمة الإحتمالية تساوي(0.04).

وخلصت هذه الدراسة إلى وجود إنتاج عالي لإنزيم البيتالاكتاميز واسع الطيف في المستشفيات، وكانت الكليبسيلة الأكثر إنتاجا.نحتاج إلى إختبارات أخرى حديثة للكشف عن إنزيم البيتالاكتاميز واسع الطيف وإنزيم أمبلار (ج) كإختبارات تأكيدية. يجب التحكم في استخدام المضادات الحيوية في المستشفيات والوقاية من الأمراض عن طريق تدابير أخرى غير استخدام المضادات الحيوية للحد من مقاومة المضادات الحيوية.

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INTRODUCTION

1.1.Introduction

As a normal part of life, all people are exposed to pathogens due to many factors such as ecological, environmental, or demographic factors that place them at increased contact with a previously unfamiliar microbe (Stephen, 1995). These tiny microbes and viruses are cause infectious disease. Human bodies have natural defense systems, but those systems sometimes fail to control infection. For these purpose, pharmaceutical companies have developed antibiotics, chemicals that interfere with specific life processes of pathogens. As a natural response, antibiotic resistance emerges in pathogen populations (Karl and David, 2011).

Each year in the United States, at least 2 million people acquire serious infections with bacteria that are resistant to one or more of the antibiotics designed to treat those infections. At least 23,000 people die each year as a direct result of these antibiotic-resistant infections (Tom, 2013).

There are many mechanisms that mediate bacterial resistance. One of these mechanisms is production of enzymes that inactivate the drugs, such as β-lactamases (Levinson, 2016). These family of enzymes produced by many Gram-positive and Gram-negative bacteria that inactivate β-lactam antibiotics

by opening their β-lactam ring (Lakshmi *et al*, 2014). These enzymes are classified as serine β-lactamase when they have a serine radical or as metallo-β-lactamases (MBLs) when they have zinc ion at the enzyme's active site (Michael *et al*, 2012). The simplest classification is by protein sequence, where by the β-lactamases are classified into four molecular classes, A, B, C, and D, based on conserved and distinguishing amino acid motifs (Karen and George, 2010). Classes A, C, and D comprise evolutionarily distinct groups of serine enzymes, and class B contains the zinc types (David, 1995). Because of the increased spectrum of bacterial activity against new expanded-spectrum β-lactams, particularly the cephalosporins, the β-lactamases are called extended-spectrum β-lactamases or ESBL. In general, ESBLs are β-lactamases that are capable of conferring resistance to penicillin and all generation of cephalosporins, by hydrolyzing these antibiotics (Merlyn and Cynthia, 2017). These enzymes are plasmid borne and they have evolved from point mutations which altered the configuration of the active site of the original and long known β-lactamases, which have been designated as TEM-1, TEM-2, and SHV-1 (Abd Elrahman *et al*, 2017). In Sudan, there are 60 to 80% of *E.coli* and *K. pneumonia* isolates encountered in two teaching hospitals in Khartoum are active producers of extended-spectrum beta-lactamase (ESBL) and the prevalence of Methicillin-resistant *Staphylococcus*

*aureus (*MRSA) infections in this country is ranging between 30 to 80%, and the situation is further aggravated by the emergence of 3 to 5% of resistance among *Pseudomonas* spp even to carbapenems which are uniquely resistant to hydrolysis by most β-lactamases (Sami, 2014).

There are many tests that used for detection of β-Lactamases. Most use chromogenic cephalosporins which is very specific and including nitrocefin test, iodometric test and acidimetric test. Double disc synergy test (DDST) and modified double disc method (MDDS) also can be used (David and Derek, 2001).

1.2. Rationale

The emergence of resistance is a major problem worldwide in antimicrobial therapy. Infections caused by resistant microorganisms often fail to respond to the standard treatment resulting in prolonged illness, higher healthcare expenditures, and greater risk of death (Jun *et al*, 2015)

β-Lactamases are the commonest cause of bacterial resistance to β-lactam antimicrobial agents. Their spread destroyed the utility of benzylpenicillin against *staphylococci* and has hugely undermined that of ampicillin against *enterobacteria* and *Haemophilus* and *Neisseria* spp (David, 1995).

In Sudan injectable antibiotics are limited to cephalosporins, vancomycin, fluoro quinolones, aminoglycosides and carbapenems. Standard injectable antibiotics such as ampicillin, cloxacillin and macrolides for treating common infections are not available, clinicians use oxyiminocephalosporins or vancomycin to treat these infections (Awad, 2015), therefor situation in Sudan became very grim due to over use of cephalosporin even when compared with some neighbouring countries, and there is little information about this problem.

This study was done to detect beta-lactamase in different clinical specimens.

1.3. Objectives

1.3.1. General objective

To detect extended spectrum β-lactamase and Amp-C β-lactamase among Gram negative bacteria isolated from infected patients in different Hospitals in Khartoum stat.

1.3.2. Specific objectives

1. To isolate and identify bacterial species from urine and wound swabs specimens.
2. To perform antimicrobial susceptibility testing by using modified Kirby-Bauer disc diffusion method.
3. To detect ESBLs in isolated bacteria using double disc synergy test (DDST).
4. To detect Amp-C β-lactamase in isolated bacteria using disc approximation test.
5. To correlate between the presence of (ESBLs, Amp-C) β-lactamase and gender of patients and age.

LITERATURE REVIEW

2.1. Antimicrobial agents

The antimicrobials are the agents that kill or inhibit the growth of microorganisms, This microbial agent may be a chemical compounds and physical agents. These agents interfere with the growth and reproduction of causative organisms like bacteria, fungi, parasites, virus. They are classified in deffirent ways according to microorganisms agent which they are used, their ability to kill or inhibit the microorganism, source, their site of action and usage and according to chemical structur and mechanisme of action(Daniel, 2013). The discovery of antimicrobials is one of the great advances in medicine and their use has substantially reduced morbidity and mortality worldwide (Levinson, 2016). Several key steps must be completed for an antimicrobial agent to successfully inhibit or kill the infecting microorganism. First, the agent must be in an active form. Second, the antibiotic must also be able to achieve sufficient levels or concentrations at the site of infection. The remaining steps relate to direct interactions between the antibacterial agent and bacterial cell, adequate binding of the target results in disruption of certain cellular processes leading to stop of bacterial cell growth, and depending on the antimicrobial agent's mode of action (Stuart, 2005).

2.2. Antimicrobial drug resistance

Antibiotic resistance is a worldwide problem. Many forms of resistance spread with remarkable speed. World health leaders have described antibiotic resistant microorganisms as “nightmare bacteria” that “pose a catastrophic threat” to people in every country in the world (Tom, 2013). There are many

different mechanisms by which microorganisms might exhibit resistance to drug, like production of enzymes that destroy the active drug, also microorganisms can change their permeability to the drug, and develop an altered structural target for the drug (Betty *et al*, 2007). Multidrug- resistance pump or efflux pump also mediate bacterial resistance to drugs. This pump is imports protons and, in an exchange-type reaction, exports a variety of foreign molecules including certain antibiotics, such as tetracycline (Levinson, 2016).

2.2.1. Resistance to β-lactam antibiotic

Penicillin, penicillin derivatives, cephalosporins, cephamycins, carbapenems, monobactams are classified as β-lactam antibiotics. All possess an essential four-membered lactam ring that may be fused to form bicyclic ring structures or may exist as isolated rings. These antibiotics interfere with the final stage of cell wall (peptidoglycan) synthesis by inhibiting the bacterial enzymes, transpeptidases, and carboxypeptidases that catalyze the reactions of peptidoglycan synthesis. These enzymes commonly called penicillin-binding-protein (Sabiha, 2001). The production of β-lactamase by bacteria is one of the most efficient and prevalent mechanisms of resistance to β-lactam antibiotics (Christian and Roger, 2000).

2.2.2. Bacterial β-lactamases

Bacterial β-lactamases are members of an enzyme family that deactivate the effect of β-lactam antibiotics such as penicillins, monobactams, and carbapenems by attacking their β-lactam rings (Hakime *et al*, 2015). These enzymes also vary in their spectrum of substrates, that is not all β-lactams are susceptible to hydrolysis by every β-lactamase. For example, *staphylococcal* β-lactamase can readily hydrolyze penicillin and penicillin derivatives, such

as ampicillin, mezlocillin, and piperacillin, but this enzyme cannot effectively hydrolyze many cephalosporin or imipenem (Betty *et al*, 2007).

2.2.3. Classification of β-lactamase

Beta-lactamase are classified into Ambler and Bush Jacopy-Mederiros, and The most widely used is the Ambler classification. That divides β-lactamases into four classes (A, B, C and D) based upon their amino acid sequences. Ambler originally specified two classes: class A, the active-site serine β-lactamases; and class B, the metallo-β-lactamases that require a bivalent metal ion, usually Zn, for activity. Class C its members are also known as the 'Amp-C' β-lactamases. Another class of serine β-lactamases, familiarly known as the OXA β-lactamases, was found to bear little resemblance to either 4class A or class C and was designated class D (Barry, 2005). Classes A and D β-lactamases are representing the largest group of β-lactamases, due primarily to the increasing identification of ESBLs during the past 20 years. Class C (Amp-C) β-lactamases is called cephalosporinases which encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms, and they are more active on cephalosporins than benzylpenicillin and are usually resistant to inhibition by clavulanic acid and active on cephamycins, such as cefoxitin, also they are have high affinity for aztreonam .Class B β-lactamases (metallo- β-lactamases) they were once distinguished primarily by their ability to hydrolyze carbapenems, but some serine β -lactamases now have also acquired that ability. They are having poor affinity or hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam, but they can be inhibited by metal ion chelators such as EDTA, dipicolinic acid (Karen and George, 2010)

2.2.4. Detection of β-lactamases

2.2.4.1. Direct tests for β-lactamases activity

Direct β-lactamase tests are mostly used for fastidious Gram-negative species (*Haemophilus influenzae, Moraxella catarrhalis and Neisseria* spp) where few enzyme types occur, *staphylococci* and Gram-negative bacilli. Numerous β-lactamase detection tests have been devised but rather fewer are convenient for routine use. Most use chromogenic cephalosporins test, or link the hydrolysis of penicillin to a colour change mediated by iodine or a pH indicator, and there are three type of this test (David and Derek, 2001).

2.2.4.1.1. Nitrocefin test

Colonies of the test isolate is suspended in nitrocefin solution (which containing of nitrocefin powder dissolved in dimethylsulphoxide) β-lactamase activity is indicated by change in the color from yellow to red. It provides the most sensitive test for most β-lactamases, exceptions being staphylococcal penicillinase and an uncommon plasmid-mediated enzyme of *haemophili* (David and Derek, 2001).

2.2.4.1.2. Iodometric tests

Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolourizing the starch–iodine complex. This reaction can be exploited to detect β-lactamase activity in tubes or on paper strips. Activity of β-lactamase is demonstrated by decolorization of the iodine within 5 min. These tests are particularly sensitive for staphylococcal penicillinase, but are less sensitive than nitrocefin for most of the β-lactamases from Gram-negative bacteria (David and Derek, 2001).

2.2.4.1.3. Acidimetric tests

Hydrolysis of the β-lactam ring generates a carboxyl group, acidifying unbuffered systems. The resulting acidity can be tested in tubes or on filter papers. Changing in the color from red in to yellow indicates β-lactamase activity. The method is useful for tests on *H. influenzae* and *Neisseria gonorrhoeae* (David and Derek, 2001).

2.2.4.2. 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 (David and Derek, 2001).

2.2.4.2.1. Double disc synergy test (DDST)

Double disc approximation test is performed for ESBL detection. Synergy is determining between a disc of amoxicillin-clavulanate (20 μg/10 μg) (augmentin) and a 3 disc of each third-generation cephalosporin test antibiotic placed at a distance from center to center on a Mueller-Hinton Agar (MHA) plate swabbed with the test isolate, any extension showing at the edge of the inhibition zone of cephalosporin toward the augmenting disc is interpreted as positive for ESBL production (Ravi *et al*, 2011).

2.2.4.2.2. Modified double disc synergy test (MDDST)

The ESBL productions testing by using a disc of amoxicillin-clavulanate (20/10 μg) along with four cephalosporins; third generation cephalosporin (cefotaxime, ceftriaxone, cefpodoxime) and fourth generation cephalosporin (cefepime). A disc which contained amoxicillin-clavulanate (20/10 μg) is place in the center of the plate swabbed with the test isolate, the discs of third

generation cephalosporin and fourth generation cephalosporin are placed 15mm and 20mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc and any distortion or increase in the zone towards the disc of amoxicillin-clavulanate is consider as positive for the ESBL production (Jaspal *et al*, 2013).

2.2.4.2.3. Disc approximation test

This test is used to detect of Amp-C β-lactamase enzyme, it is performed by using ceftazidime 30-µg, imipenem10-µg and amoxicillin-clavulanate30-µg discs. The production of this enzyme is detect by observation of any blunting or flatting of zone between the ceftazidime disc and inducing substrates (Gunman, Vibhor and Purva, 2014)

2.2.4.2.4. confirmatory combination disc diffusion test

These depend on comparing the zones given by discs containing an extended-spectrum cephalosporin with and without clavulanic acid. If an ESBL is produced, the zones are enlarged for the discs containing the inhibitor. (Ravi *et al*, 2011).

2.2.4.2.5. Disc placement method

A novel disc placement is performed for ESBL detection and AmpC type β-lactamase at the same time. Seven antibiotics discs are used in this test, viz imipenem(10µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), ceftazidime + clavulinic acid (30/10 µg), and aztreonam (30 µg).Imipenem, the inducer of β-lactamase, is place in the center, and the remaining discs are place as per instructions of this test, and the test is interpreted as either the isolate is ESBL producer, inducible AmpC producer

or a derepressed mutant or have multiple mechanisms of resistance as suggested (Neena *et al*, 2012).

2.3. Previous studies

In 2013, study was aimed to determination of ESBLs and AmpC production in uropathogenic isolates of *Escherichia coli* and Susceptibility to Fosfomycin. A total number of 150 *E. coli* isolates were studied, ESBL detection was done by double disc synergy and Clinical Laboratory Standard Institute (CLSI) method (using Ceftazidime and Ceftazidime + Clavulanic acid combination discs). AmpC screening was done using cefoxitin disc and confirmation was done using cefoxitin/cefoxitin-boronic acid discs, and Fosfomycin susceptibility was determined by disc diffusion and E-test methods. ESBLs production was seen in 52.6% of isolates and AmpC production was seen in 8% of isolates and all AmpC producers were also found to be ESBLs positive. ESBLs positive isolates were found to be more drug resistant than ESBLs negative isolates. All the strains were found to be fosfomycin sensitive (Varsha *et al*, 2013).

Another study also aimed to determine the prevalence of ESBL in strains of *K. pneumonia* isolated from different clinical samples. In This study was found 76.9% (100) of the isolates were resistant to cefuroxime, cefepime and cefazolin, 69.23% (90) were resistant to cefotaxime, and 46.15% (60) were resistant to cefoxitin. Extended spectrum β-lactamase was detected in 53.8% (70) of *Klebsiella*. *pneumonia*. All *Klebsiella pneumonia* isolates were resistant to ampicillin followed by both piperacillin and mezlocillin 92.30% (120). *Klebsiella pneumonia* isolates showed high sensitivity to imipenem

(15.38%) (20), followed by ertapenem, tetracycline, tigecycline piperacillin/tazobactam and amikacin (23.07%) (Khalid and Ahmed, 2014).

A Serbian study conducted through 2014, was performed to detect of production and identification of β-lactamase type phenotypically in *Enterobacteriaceae* isolated from different clinical specimens from patients hospitalized in the Clinical Center of Serbia. A total of 172 *Enterobacteriaceae* strains were isolated. Further testing was performed on 54/145 (37.2%) strains showing decreased susceptibility to β-lactam antibiotics: 13/85 (15.3%) Escherichia coli, 31/46 (67.4%) *Klebsiella pneumonia* and 10/14 (71.4%) *Proteus mirabilis*. Among them, 40/145 (27.6%) strains produced extended spectrum β-lactamases (ESBLs), 9/145 (6.2%) AmpC, 1/145 (0.7%)-K1 β-lactamase and 4/145 (2.8%) *carbapenemases. Carbapenemases* were predominantly detected in *K. pneumonia* (75%) (Cirkovic *at el,* 2014).

In Indian study was conducted in Tertiary Care Hospital, which aimed to detection of extended spectrum β-lactamase and Amp-C β-lactamase producing clinical isolates, found among the 148 isolates, 82 (55.40%) were ESBL producers, and 115 (77.70%) were Amp-C β-lactamases producers. Co-existence of ESBL and AmpC was observed in 70 (47.29%) isolates. *Escherichia coli* was the most common ESBL and AmpC β-lactamase producer. All ESBL producers were highly resistant to ciprofloxacin (83.10%), co-trimoxazole (95.27%), and gentamicin (89.18%). However, these Bacterial strains were sensitive to imipenem 146 (98.64%) and piperacillin/tazobactam 143 (96.62%) (Sageerabanoo *at cl,* 2015).

In Sudan, study was aimed to determine the prevalence of extended spectrum β-lactamase and Amp-C producing bacteria in a Sudanes tertiary hospital. A total of 150 Gram negative isolates were collected. Seventy-eight (52%) strains produced ESBLs only whereas 4 (2.7%) organisms produced AmpC β-lactamase and six (4%) co-produced ESBLs and AmpC β-lactamases. *K. pneumonia* and *E. coli* were the most common ESBL-producers among all clinical isolates accounting for (31/37) 83.8% and (39/63) 61.9% respectively. Inducible AmpC beta-lactamase production was demonstrated in 17 strains (11%), 13 of which were *P. aeruginosa*. Sixty-three per cent of ESBL producing isolates were resistant to ciprofloxacin, while only 44% of ESBL negative isolates were resistant to ciprofloxacin. There was no association between ESBL production and co-resistance to other antibiotics. Only four isolates (2.7%) were resistant to meropenem (Awad, 2015).

Another study in Sudan was aimed to detection of extended spectrum β-Lactamases phenotypically (ESBL) among Gram negative uropathogens reveals highly susceptibility to imipenem. Isolated bacteria were tested for antibiotic susceptibility and ESBL screening using modified Kirby- Bauer method and Double Disc Synergy Test (DDST) respectively. A total of 33 of bacteria were isolated from urine and the most prevalent isolates were Gram negative bacteria 18/33(54.5%). Among gram-negative bacteria, isolates of *Escherichia coli* were the most prevalent accounting (12/18, 66.6%) followed by *Klebsiella pneumoniae* (4/18, 22.2%) and *Klebsiella oxytoca* (2/18, 11.1%). ESBL was detected in 8/18 (44.4%) of the Gram-negative isolates. Imipenem was the most susceptible antibiotic for ESBL-producer and non-ESBL producer Gram negative isolates, accounting 100% susceptibility for both bacterial groups (Mohammed and Abass, 2019).

MATERIALS AND METHODS

3.1. Study design

This was descriptive cross-sectional study.

3.1.2. Study area

This study was done in Khartoum state. Clinical specimens were collected from Omdurman Teaching Hospital, Asia Hospital and Omer Sawy Police Hospital. The experimental work was carried out in the research lab, Sudan University of Science and Technology.

3.1.3. Study duration

This study was conducted during the period from March 2018 to February 2019.

3.2. Study population

All patients attended the above-mentioned hospitals and suspected to have bacterial infection.

3.2.1. Inclusion criteria

Patients presented with wound infection and urinary tract infection were included in this study.

3.2.2. Exclusion criteria

All patients who had taken antimicrobials within the past two weeks or patients with other disease or refused to participate in this study were excluded from this study

3.3. Sample size

A total of 100 patients were included in this study.

3.4. Experimental work

3.4.1. Collection of specimens

Specimens were collected from patients using standard microbiological methods. Mid-stream urine was collected in sterile, dry, wide-necked, leak-proof container. exudate from wound was collected using sterile cotton-wool swab. The specimens were immediate delivered to the laboratory for culture and sensitivity.

3.4.2. culture

Specimens were cultured on blood agar, MacConkey agar and cystine lactose electrolyte deficiency (CLED) medium then incubated aerobically overnight at 37 °C. The isolates were streaked on nutrient agar to check their purity after overnight incubation at 37 °C.

3.4.3. Identification of bacteria

3.4.3.1. Gram stain

Smear was prepared by emulsifying a small portion of bacterial colony in a drop of normal saline and spread evenly on a clean slide. Then smear was allowed to air-dry on a safe place-protected from the dust and sun light. The smear was fixed by rapidly pass the slide smear uppermost three times through the flame of a spirit lamp or pilot flame of a Bunsen burner. Then allow the smear to cool before staining it.

The fixed smear was covered with crystal violet stain for 30–60 seconds. The stain was rapidly washed off with clean tap water. All the water was tipped

off and cover the smear with Lugol's iodine for 30–60 seconds. The iodine was washed off with clean water and rapidly decolorized (few seconds) with acetone–alcohol. Wash immediately with clean water. Then the smear was covered with neutral red stain for 2 minutes. The neutral red was washed off with clean water. The back of the slide was wiped clean, and place it in a draining rack for the smear to air-dry. The dried smear was examined microscopically, first with the 40X objective to check the staining and to see the distribution of material, and then with the oil immersion objective to report the bacteria (Cheesbrough, 2009).

3.4.3.2. Biochemical tests for Gram negative rod

3.4.3.2.1. Oxidase test

This test used to determine the presence of bacterial cytochrome oxidase by using the oxidation of the substrate tetramethyl-p-phenylenediamine dihydrochloride to indophenol.

A piece of filter paper was placed in a clean petri dish and 2 drops of freshly prepared oxidase reagent was added. A colony of the test organism was removed by using a piece of stick and smear it on the filter paper, a positive result was indicated by developing of dark-purple colour within few seconds. (Chesbrough, 2009).

3.4.3.2.2. Sugar fermentation and production of H_2S and gas

Kligler iron agar was used to determine whether a Gram-negative rod utilizes glucose and lactose or sucrose fermentation and forms hydrogen sulphide, and consist of phenol red and ferrous sulphate which serve as indicators of acidification and hydrogen sulphate (H_2S) formation, respectively. If the entire of medium becomes acidic (yellow) and the slant reverts alkaline (red) state, that indicate fermentation of glucose. When in addition to glucose,

lactose and/or sucrose are fermented, the large amount of fermentation products formed in the slant and become acidic(yellow) (Betty *et al*, 2007).

3.4.3.2.3. Citrate utilization test

The test is based on the ability of an organism to use citrate as its only source of carbon. After preparation of Simmon's citrate agar in slope in bijou bottles, using a sterile straight wire, first streak the slope with a saline suspension of the test organism and then stab the butt (Cheesbrough, 2009).

3.4.3.2.4. Urease test

The test organism is cultured in a medium which contains urea and the indicator phenol red. When the strain is urease producing, the enzyme will break down the urea (by hydrolysis) to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in colour of the indicator to pink-red (Cheesbrough, 2009).

3.4.3.3. Susceptibility of bacterial isolates to antibiotics

According to the National Committee for Clinical Laboratory Standards (NCCLS), The modified Kirby–Bauer disc-diffusion method was performed (Vandepitte *et al*, 2003) as following:

3.4.3.3.1. Antimicrobial discs

Antibiotics tested were ceftazidime (30µg), imipenem(10µg) ciprofloxacin (5µg), gentamicin (10µg), amikacin (30µg). Nitrofurantoin (300µg) was added for urinary isolates. Were obtained from (Bioanalyse- Turkey).

3.4.3.3.2. Preparation of inoculum

The inoculums were prepared from the primary culture plate, touch with a loop the tops of each of 3–5 colonies, of similar appearance, of the organism to be tested. This growth was transferred to a tube of saline. Compare the tube

with the 0.5 turbidity standard (McFarland standard) and adjust the density of the test suspension to that of the standard by adding more bacteria or more sterile saline (Vandepitte *et al*, 2003).

3.4.3.3.3. Seeding of the plates

The plates were inoculated by dipping a sterile swab into the inoculum. The excess inoculum was removed by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. Streak the swab all over the surface of the medium three times, rotating the plate through an angle of 60° after each application. Finally, pass the swab round the edge of the agar surface. Leave the inoculum to dry for a few minutes at room temperature with the lid closed.

3.4.3.3.4. Application of antibiotics

The antimicrobial discs were placed on the inoculated plates by using a pair of sterile forceps, the discs were spaced evenly, approximately 15 mm from the edge of the plate, and 1 disc placed in the centre of the plate. Each disc should be gently pressed down to ensure even contact with the medium, then the plates should be placed in an incubator at 35 °C within 30 minutes of preparation for 24 hours.

3.4.3.3.5. Reading of the zones of inhibition

After overnight incubation, the diameter of each zone (including the diameter of the disc) should be measured and recorded in mm, the measurements were made with a ruler on the under-surface of the plate without opening the lid.

3.4.3.3.6. Interpretation of the results

The zones size of each antimicrobial was reported by using the interpretative Chart.

3.4.3.3.7. Testing organisms for quality control

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.

3.4.3.4. Detection of β-lactamase

3.4.3.4.1. Screening test

All isolates were found resistant to third generation cephalosporin (ceftazidime-30 μg) were regarded as potential ESBL or Amp-C producers and they were further tested.

3.4.3.4.2. Detection of ESBLs

Double disc synergy test (DDST) was used to detect ESBLs. A synergy was determined between a disc of amoxicillin-clavulanate 30-μg (augmentin) and a 30-μg disc of third-generation cephalosporin (ceftazidime and cefotaxime) test antibiotic placed at a distance of 20 mm from center to center on a Mueller-Hinton Agar (MHA) plate after swabbed with the tested isolate. Clear extension of the edge of the inhibition zone of cephalosporin toward the augmentin disc was interpreted as positive for ESBL production.

3.4.3.4.3. Detection of Amp-C β-lactamase

Disc approximation test was used to detect Amp-C β-lactamase. It was performed by using imipenem10-μg and amoxicillin-clavulanate30-μg (as inducing substrates) and ceftazidime 30- μg discs. A 30 μg ceftazidime disc was placed at the center on the Mueller–Hinton agar seeded with the test organism, then a 10 μg imipenem and 30 μg amoxicillin-clavulanate discs were placed at a distance of 20 mm from ceftazidime disc. The plate inverted

and incubate over night at 35°C. After overnight incubation, the plate was examined for any obvious blunting or flattening of the zone of inhibition between the ceftazidime disc and the inducing substrates. Any blunting or flattening of the zone, consider as a positive result for Amp-C production.

3.4.3.4.4. Quality control

All test used to detect ESBLs and Amp-C β-lactamase were checked for quality using standard control ESBL and Amp-C negative strain of *Escherichia coli* ATCC 25922.

3.5. Statistical analysis

Statistical package for social science (SPSS) and computer software version 16.0 was used to analyses data to check the statistical significance the p-value considered significant was < (0.05)

RESULTS

A Total of 100 clinical specimens were collected from patients in Omdurman Teaching Hospital, Asia Hospital and Omer Sawy Military Hospital. The patients investigated were 39(39%) males, and 61(61%) were females as shown in **table** (4-1). A thirty-seven of specimens (37.0%) was collected from patients aged 10-30 years, and the same number 37(37.0%) of these specimens collected from patient aged 31-60 years, while the fewest number of specimens 26(26.0%) was collected from patients aged 61-95 years as shown in **table** (4-2).

A fifty-eight (58%) of specimens were gave bacterial growth, while (42%) specimens showed no bacterial growth. Urine was the major source of the bacterial isolates 42(72.41%), followed by wound swab 16(27.59%). The most common species isolated from specimens were *Klebsiella pneumoniae* 26 (44.8%) and *Escherichia coli* 14(24.1%) as shown in **table** (4-3). All isolated bacteria were subjected to antimicrobial sensitivity test. The results were found to be susceptible to Amikacin (89%) follow by Gentamicin (70%), Imipenem (74.1%) and Ciprofloxacin (48.3). Forty-five (77.6%) of isolates were resistant to Ceftazidime as showing in **table** (4-4). Nitrofurantoin was added for urinary isolates, and found to be susceptible to all these isolated microorganisms 42(100,0%) as shown in **table** (4-5).

The result revealed that 10(17.2%) of isolates were ESBLs positive. These were 5(8.6%) *Klebsiella pneumoniae,* 3(5.2%) *Escherichia coli* and 2(3.4%) *Proteus spp* as in **table** (4-6), and only 4 (6.9%) of isolates were Amp-C β-lactamase positive. These were 2 (3.4%) *Pseudomonas aeruginosa*, 1(1.8%) *Klebsiella pneumoniae* and 1(1.8%) *Proteus spp* as in **table** (4-7). The

association between ESBL producer organisms and gender was found statistically insignificant (*p*. value = 0.635) as shown in **table** (4-8). While the association between Amp-C β-lactamase producer organisms and gender was found statistically significant (*p*. value = 0.03) as shown in **table** (4-9). The association between ESBL producer organisms and age groups was found statistically insignificant (*p*. value = 0.09) as shown in **table** (4-10), While the association between Amp-C β-lactamase producer and age groups was found statistically significant (*p*. value = 0.04) as shown in table (4-11).

Table (4-1): Frequency of enrolled patients according to the gender

Gender	Number	Percentage
Male	39	(39.0%%)
Female	61	(61.0%)
Total	100	(100.0%)

Table (4-2): Frequency of clinical specimens according to the age groups

Age group	Specimens		Total
	Urine	wound swab	
(10-30) years	26 (26.0%)	11.0 (0%)	37 (37.0%)
(31-60) years	25(25.0%)	12(12.0%)	37 (37.0%)
(61-95) years	23 (23.0%)	3 (3.0%)	26 (26.0%)
Total	74 (74.0%)	26 (26.0%)	100 (100.0%)

Table (4-3) Frequency of clinical isolates

Isolate	Frequency	Percentage
Klebsiella pneumoniae	26	44.8%
E. coli	14	24.1%
Proteus spp	11	19.0%
Pseudomonas Aeruginosa	6	10.3%
Citrobacter spp	1	1.8%
Total	58	100.0%

Table (4-4) Antimicrobial susceptibility patterns of the isolated microorganisms

Organisms	*E. coli*		*Citrobacter spp*		*Klebsiella pneumoniae*		*Proteus spp*		*Pseudomonas aeruginosa*	
Antibiotic	S	R	S	R	S	R	S	R	S	R
Ceftazidime	5 8.6%	9 15.5 %	0 0%	1 1.8%	3 5.2%	23 39.7 %	5 8.6%	6 10.3 %	0 0%	6 10.3 %
Imipenem	12 20.7 %	2 3.4%	1 1.8%	0 0%	18 31.0 %	8 13.8 %	9 15.5 %	2 3.4%	3 5.2%	3 5.2%
Ciprofloxacin	4 6.9%	10 17.2 %	0 0%	1 1.8%	16 27.6 %	10 17.2 %	5 8.6%	6 10.3 %	3 5.2%	3 5.2%
Gentamicin	10 17.2 %	4 6.9%	1 1.8%	0 0%	20 34.5 %	6 10.3 %	8 13.8 %	3 5.2%	2 3.4%	4 6.9%
Amikacin	13 22.4 %	1 1.8%	0 0%	1 1.8%	24 41.2 %	2 3.4	10 17.2 %	1 1.8%	5 8.6%	1 1.8%

Key: S= Sensitive

R= resistance

Table (4-5) Antimicrobial susceptibility of urinary isolate to nitrofurantoin

Microorganism	**Nitrofurantoin**		**Total**
	S	R	
E. coli	13 (31.0%)	0 (0%)	13 (31.0%)
Klebsiella Pneumoniae	22 (52.3%)	0 (0%)	22 (52.3%)
Proteus Spp	5 (11.9%)	0 (0%)	5 (11.9%)
Pseudomonas Aeruginosa	2 (4.8%)	0 (0%)	2 (4.8%)
Total	42 (100.0%)	0 (0%)	42 (100.0%)

Key: S= Sensitive
R= Resistance

Table (4-6) Frequency of ESBLs positive and negative isolates

Microorganism	**ESBLs**		**Total**
	Positive	**Negative**	
E. coli	3 (5.2%)	11 (19%)	14 (24.1%)
Citrobacter spp	0 (0%)	1 (1.8%)	1 (1.8%)
Klebsiella pneumoniae	5 (8.6%)	21 (36.2)	26(44.8%)
Proteus spp	2 (3.4%)	9 (15.5%)	11(19.0%)
Pseudomonas aeruginosa	0 (0%)	6 (10.3%)	6 (10.3%)
Total	10 (17.2 %)	48 (82.8%)	58 (100.0%)

Table (4-7) Frequency of Amp-C β-lactamase positive and negative isolates

Microorganism	Amp-C		Total
	Positive	Negative	
E.coli	0(0%)	14(24.1 %)	14(24.1%)
Citrobacter spp	0 (0%)	1 (1.8%)	1 (1.8%)
Klebsiella pneumoniae	1 (1.8%)	25(43.1 %)	26(44.8%)
Proteus spp	1 (1.8%)	10(17.2 %)	11 (19.0%)
Pseudomonas aeruginosa	2(3.4%)	4(6.9%)	6 (10.3%)
Total	4 (6.9%)	54 (93.1)	58(100.0%)

Table (4-8) Association between ESBLs and gender

Gender	ESBLs		Total	P. value
	Positive	Negative		
Male	5(8.6%)	24(41.4%)	29(50.0%)	0.635
Female	5 (8.6%)	24 (41.4%)	29(50.0%)	
Total	10 (17.2%)	48 (82.8%)	58(100.0%)	

Table (4-9) Association between Amp-C β-lactamase and gender

Gender	Amp-C		Total	P. value
	Positive	Negative		
Male	4 (6.9%)	25 (43.1%)	29 (50.0%)	0.038
Female	0 (.0%)	29 (50.0%)	29 (50.0%)	
Total	4 (6.9%0	54 (93.1%)	58 (100.0%)	

Table (4-10) Association between ESBLs and age group

Age group	ESBLs		Total	p. value
	Positive	negative		
(10-30) years	3 5.2%	17 29.3%	20 34.5%	0.09
(31-60) years	2 3.4%	22 37.9%	24 41.4%	
(61-95) years	5 8.6%	9 15.5%	14 24.1%	
Total	10 (17.2%)	48 (82.8%)	58 (100.0%)	

Table (4-11) Association between Amp-C β-lactamase and age group

Age group	Amp-C		Total	P. value
	Positive	Negative		
(10-30) years	0 (0%)	20 (34.5%)	20 (34.5%)	0.04
(31-60) years	4 (6.9%)	20 (34.5%)	24 (41.4%)	
(61-95) years	0 (0%)	14 (24.1%)	14 (24.1%)	
Total	4 (6.9%)	54 (93.1%)	58 (100.0%)	

DISCUTION

5.1. Discussion

The emergence of resistance is a major problem worldwide in antimicrobial therapy. Infections caused by resistant microorganisms often fail to respond to the standard treatment resulting in prolonged illness, higher healthcare expenditures, and greater risk of death. β-Lactamases are the commonest cause of bacterial resistance to β-lactam antimicrobial agent (Jun *et al,* 2015).

This study was done to determine β-lactamas enzyme in bacteria isolated from different clinical specimen, it found that the most frequent isolates were *Klebsiella Pneumoniae* (26) and *Escherichia coli* (14), and the prevalence of ESBLs was10(17.2%) detected in 5 (8.6%) *Klebsiella* ,3 (5.2%) *E. coli* and 2(3.4%) *Proteus.* This result was consistent with other reports in Sudan, it is higher than that reported in study conducted in Omdurman Military Hospital in Khartoum state by (Alamin and Hamed, 2013) who found 5(11.9%) of isolated bacteria were ESBLs positive found in 2 *Klebsiella*, 2 *E. coli* and 1 *Proteus* isolates. The prevalence of ESBL in this study was closely similar to result obtained from Elsara (2015) who found (21.6%) of Gram-negative bacteria were ESBL producer, and 60(63.1%) of 95 isolated bacteria were

showed resistant to third-generation cephalosporin and they were tested for the ESBL production by double disc synergy method. The overall of ESBL producer only 22(36.67%) of isolates. As already reported, 45(77.6%) of isolated organism in the present study were showed resistant to third-generation cephalosporin (ceftazidime), while the ESBL producer organism only 10(17.2%) of isolate. The routine susceptibility tests by clinical laboratories unable to detect ESBL producer strains. The prevalence of Amp-C β-lactamase producing organisms in this study was only 6.9% of isolated bacteria which higher than the result obtained from (Awad, 2015) who found only 2.7% of organisms produced Amp-C β-lactamase.

The association between ESBLs producer organisms and gender was found insignificant statistically (p. value=0.635), in agreement with the result of study conducted in Chad and obtained by (Oumar *et al*, 2019) who they found gender not significantly associated with ESBLs presence (p. value=0,07).

5.2. Conclusion

This study concluded that:

- There is high ratio of ESBLs among isolated bacteria and the most producer was *Klebsiella pneumonia.*

5.3. Recommendations

The following points are highly recommended:

- Advanced technique such as PCR test for detection of ESBL and Amp-C β-lactamase is required to confirm the results of this study.
- Antibiotic use should be controlled in hospitals to prevent increasing in multidrug resistant bacteria in the future.
- Prevention of disease by measures other than the use of antibiotics to reduce antibiotic resistance.

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Appendices

Preparation of culture medium

Mueller–Hinton agar

Mueller–Hinton agar should be prepared from a dehydrated base according to the manufacturer's instructions. It was cooled to 45–50 °C and pour into the plates. Allow to set on a level surface.

Blood agar

To make about 35 blood agar plates:

Nutritious agar. . . . . .. .500 ml

Sterile defibrinated blood . . . . 25 ml

1. Prepare the agar medium as instructed by the manufacturer. Sterilize by autoclaving at 121 °C for 15 minutes. Transfer to a 50°C water bath.
2. When the agar has cooled to 50°C, add aseptically the sterile blood and mix gently but well. Avoid forming air bubbles.
   Important: The blood must be allowed to warm to room temperature before being added to the molten agar.
3. Dispense aseptically in 15 ml amounts in sterile petri dishes.
4. Store the plates at 2–8°C, preferably in sealed plastic bags to prevent loss of moisture.

MacConkey agar

This medium is best prepared from ready to use dehydrated powder, available from most suppliers of culture media.
Contents: Peptone, lactose, bile salts, sodium chloride, neutral red, agar. The medium is usually used at a concentration of 5.2 g in every 100 ml distilled water.

1. Prepare as instructed by the manufacturer. Sterilize by autoclaving at 121°C for 15 minutes.
2. When the medium has cooled to 50–55°C, mix well and dispense aseptically in sterile petri dishes.
3. Store the plates at 2–8°C preferably in plastic bags to prevent loss of moisture

Cystine lactose electrolyte deficient (CLED medium)

This medium is best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents: Peptone, Lab-Lemco powder, tryptone, lactose, L-cystinebromothymol blue, agar.

1. Prepared as instructed by the manufacturer. Sterilize by autoclaving at 121°C for 15 minutes.
2. mix well before pouring (avoid air bubbles forming). Dispense aseptically in 15 ml amounts in sterile petri dishes.
3. Store the plates at 2–8°C, preferably sealed in plastic bags to prevent loss of moisture.

Preparation of McFarland standard

1. Prepare a 1% v/v solution of sulphuric acid by adding 1 ml of concentrated sulphuric acid to 99 ml of water. Mix well.
   Caution: Concentrated sulphuric acid is hygroscopic and highly corrosive, therefore do not mouth pipette, and never add the water to the acid.
2. Prepare a 1% w/v solution of barium chloride by dissolving 0.5 g of dihydrate barium chloride ($BaCl_2.2H_2O$) in 50 ml of distilled water.
3. Add 0.6 ml of the barium chloride solution to 99.4 ml of the sulphuric acid solution, and mix.
4. Transfer a small volume of the turbid solution to a capped tube or screw-cap bottle of the same type as used for preparing the test and control inoculate.

Figure (1) In this picture showing positive result in double disc synergy tests. Discs: left, ceftazidime, 30 µg; centre, amoxicillin- clavulanate 30 µg; and right, cefotaxime 30 µg. The organism is *proteus spp*.

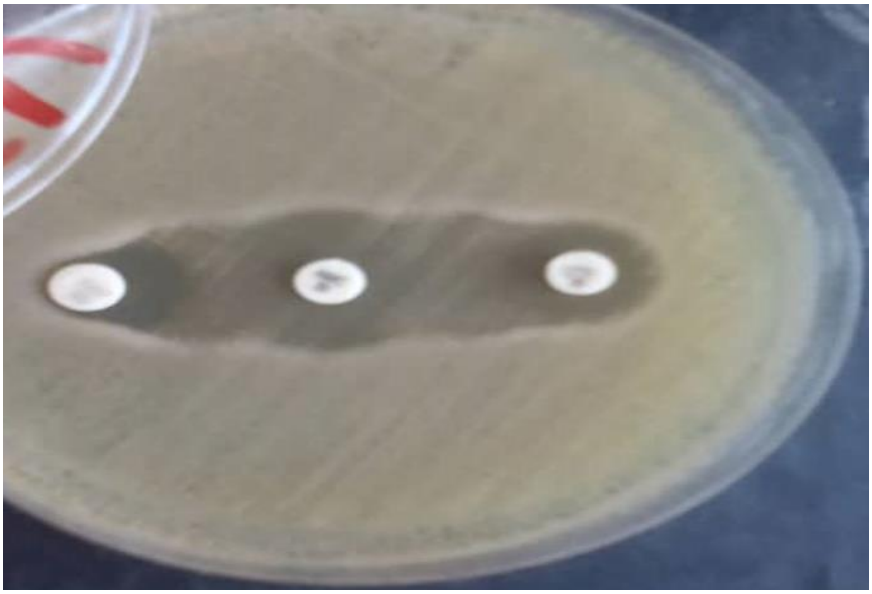

Figure (2) In this picture showing positive result in double disc synergy tests. Discs: left, ceftazidime, 30 µg; centre, amoxicillin- clavulanate 30 µg; and right, cefotaxime 30 µg. The organism is *E. coli.*

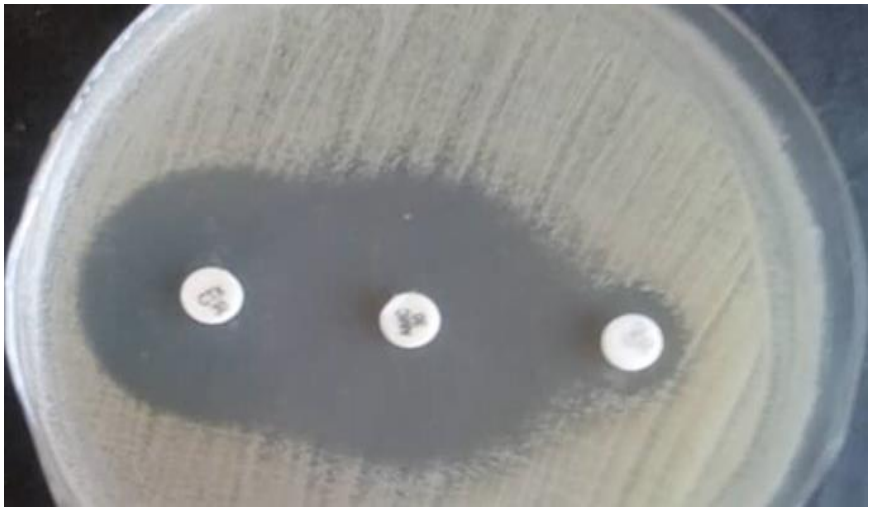

Figure (3) In this picture showing positive result in double disc synergy tests. Discs: right, ceftazidime, 30 µg; centre, amoxicillin- clavulanate 30 µg; and left, cefotaxime 30 µg. The organism is *Klebsiella pneumoniae*

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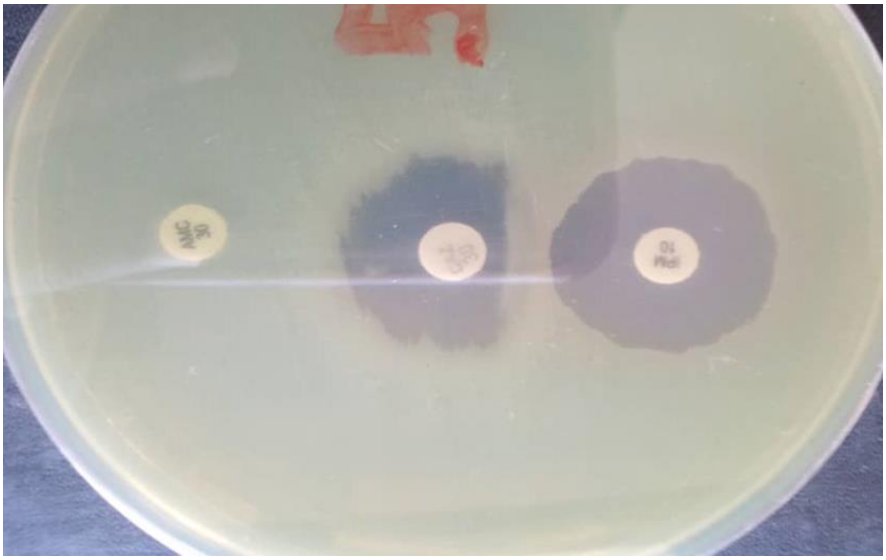

Figure (4) Detection of an Amp-C enzyme in *Pseudomonas aeruginosa*. The disc at the center is ceftazidime 30 µg, and at lift is amoxicillin-clavulanic acid 30 µg and at the right is imipenem. Note the flatting of the ceftazidime zone by the imipenem-induced enzyme.