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Phenotypic Detection of AmpC enzyme in Gram - negative Bacteria Isolated from Patients with Urinary Tract Infection in El Obaied Teaching Hospital

الكشف الظاهري عن انزيم أ ام بي سي في الباكتيريا سالبة الجرام المعزولة من المرضي بعدوى الجهاز البولي بمستشفى الأبيض التعليمي

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

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الآية

قَالَ تَعَالَىٰ:

﴿ أَوَلَيْسَ ٱلَّذِى خَلَقَ ٱلسَّمَٰوَٰتِ وَٱلْأَرْضَ بِقَٰدِرٍ عَلَىٰٓ أَن يَخْلُقَ مِثْلَهُم ۚ بَلَىٰ وَهُوَ ٱلْخَلَّٰقُ ٱلْعَلِيمُ ﴿٨١﴾ ﴾

سورة يس: الآية 81

Dedication

To my parents,

brothers, sister,

uncles, aunts

and friends,

Acknowledgement

All thanks and praises to al-Mighty ALLAH

for blessing work and breath.

My sincere thanks and acknowledgement to my supervisor Prof. Humodi Ahmed Saeed for his guidance and patience

Special thanks to laboratory team of EL-Obaied Teaching Hospital and Specialized Hospital in El-obaied

ABSTRACT

This study was done to detect the presence of AmpC enzyme among Gram negative bacteria isolated from patients of urinary tract infection attending EL-obaied Teaching Hospital. The study was conducted during the period from Jun 2018 to February 2022.

A total of 100 urine specimens were collected from patients suspected with urinary tract infection. These were 13 from males and 87 from females. The specimens were cultured on CLED agar for primary isolation. The identification of Gram-negative bacteria was done by Colonial morphology, Gram stain and biochemical tests. Antimicrobial sensitivity tests were carried out by Kirby-Bauer Disk Diffusion Method. Detection of AmpC enzyme was done by non-extract based modified three dimension test.

The result revealed that 52 Gram-negative bacteria were isolated. The isolates were 15 *Escherichia coli*, 9 *Enterobacter aerogenes*, 9 *Proteus mirabilis*, 8 *Kelbsiella pneumoniae*, 4 *Pseudomonas aeruginosa*, 3 *Citrobacter freundii*, and one *Proteus vulgaris*. AmpC enzyme was detected in 24(46.2%) out of the 52 isolates. The distribution of AmpC as follows; 4(16.7%) *Escherichia coli*, 7(29.1%) *Enterobacter aerogenes*, 1(4.2%) *Proteus mirabilis*, 2(8.3%) *Klebsiella pneumonia*, 4(16.7%) *pseudomonas aeruginosa*, 2(8.3%) *Citrobacter freundii*, 3(12.5%) *Serratia marecesns* and the 1(4.2%)*Proteus vulgaris.*

The study concluded that there is a high ratio of bacteria that are AmpC enzyme producers in UTIs patients in EL-obaied Teaching Hospital.

الخلاصة

أجريت هذه الدراسة للكشف عن وجود إنزيم أ ام بي سي بين البكتيريا سالبة الجرام المعزولة من مرضى المسالك البولية في مستشفى الأبيض التعليمي. أجريت الدراسة خلال الفترة من يونيو 2018 إلى فبراير 2022.

تم جمع ما مجموعه 100 عينة بول من المرضى المشتبه في إصابتهم بعدوى المسالك البولية. وكان هؤلاء 13 من الذكور و 87 من الإناث. تمت تربية العينات على أجار كليد للعزل الأولي. تم التعرف على البكتيريا سالبة الجرام عن طريق التعرف علي شكل المستعمرة وصبغة جرام و اختبارات الكيمياء الحيوية. تم إجراء اختبارات الحساسية لمضادات الميكروبات باستخدام طريقة كيربي باور لأنتشار القرص و تم الكشف عن إنزيم أ ام بي سي عن طريق اختبار ثلاثي الأبعاد المعدّل غير قائم على المستخلص.

أظهرت النتائج عزل 52 بكتريا سالبة الجرام. كانت العزلات 15 الإِشْرِيكِيَّةُ القولونِيَّة، ، 9 الهوائيات المعوية، 9 المُتَقَلِّبَة الرَّائِعَة، 8 الكِلِبْسِيلةُ الرِّئَوِيَّة، 4 الزائفة الزنجارية، 3 اللَّيمونِيَّة الفُرُويندِيَّة، 3 السِّرَّاتِيَّةُ الدَّابِلة و واحد المتَقَلِّبَةُ الاعْتِيادِيَّة. . تم الكشف عن إنزيم ا ام بي سي في 24 عزلة (46.2٪) من أصل 52 عزلة. توزيع أ ام بي سي على النحو التالي ؛ 4 (16.7٪) الإِشْرِيكِيَّةُ القولونِيَّة , 7 (29.1٪) الهوائيات المعوية , 1 (4.2٪) المتقلبة الرائعة, 2(8.3%) الكِلِبْسِيلةُ الرِّئَوِيَّة , 4(16.7%) الزائفة الزنجارية , 2(8.3%) اللَّيمونِيَّة الفُرُويندِيَّة , 3(12.5%) السِّرَّاتِيَّةُ الدَّابِلة و 1(4.2%)المُتَقَلِّبَةُ الاعْتِيادِيَّة.

خلصت الدراسة إلى وجود نسبة عالية من البكتيريا التي تنتج إنزيم أ ام بي سي لدي مرضى المسالك البولية في مستشفى الأبيض التعليمي.

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INTRODUCTION AND OBIECTIVES

CHAPTER ONE

1-INTRODUCTION AND OBIECTIVES

1.1 introduction

Urinary tract infections (UTIs) are the most common outpatient infections (Medina and Castillo-Pino, 2019). They are the inflammatory disorders of the urinary tract caused by the abnormal growth of pathogens. It can be asymptomatic, acute, chronic, and complicated or uncomplicated, the clinical manifestations of UTIs depend on the portion of the urinary tract involved, the etiologic organisms, the severity of the infection, and the patient's ability to mount an immune response to it (Odoki *et al*., 2019).

Antimicrobial agents are very important forms of chemotherapy that is used in the treatment of infectious diseases by killing or inhabiting the growth of microorganisms (Phillips-Jones and Harding, 2018).

The most important and widely used antimicrobials are β-lactam drugs. β-lactam antibiotics are the most often-used antimicrobials representing ~65% of antibiotic usage worldwide. The β-lactam drugs act by inhibiting bacterial cell wall biosynthesis (Palzkill, 2018). Resistance exhibited by bacterial pathogens to current antibacterial agents is now recognized to be a major global problem in the fight against infections. It is predicted that there will be 10 million deaths every year globally by 2050 unless action is taken to safeguard the effectiveness of our antibiotics (Phillips-Jones and Harding, 2018). The main mechanism of resistance to beta-lactams is the production of beta-lactamase enzymes usually encoded on mobile genetic elements that can easily be transmitted between different bacterial species or strains. those Resistant bacteria isolates causes hospital and community-

acquired infections making treatment difficult since the therapeutic options become limited. (Hashemizadeh *et al*., 2018). Resistance to β-lactam antibiotics was observed even before the introduction of the very first antibiotic, penicillin, to medical use and there are four known ways of resistance to β-lactam antibiotics: i) production of β-lactamase, ii) penicillin binding proteins that maintain the peptidoglycan structure in bacterial cell wall, iii) alteration of porin channels, and iv) initiation of efflux exporter proteins (Öztürk *et al.*, 2015).

The family of β-lactamases enzymes are produced by many Gram positive and Gram negative bacteria that inactivate the B-lactam antibiotics by opening the Beta-lactam ring. They are responsible for many causes of antimicrobial therapy failure by the hydrolysis of beta-lactam ring of these antibiotics (Lakshmi *et al.*, 2014).

The most widely used classification of those β-lactamases is the Ambler classification that divides β-lactamases into four classes (A, B, C and D) based upon their amino acid sequences with group C being AmpC enzymes (Hall and Barlow, 2005).

AmpC β-lactamases can either be plasmid or chromosomal mediated. *Citrobacter freundii, Enterobacter cloacae, Morganella morganii, Hafnia alve*i, and *Serratia marcescens* are organisms having chromosomally mediated AmpC β-lactamases. While the plasmid-mediated AmpC cephalosporinases were detected in the late 1980s that appear to be genetic descendants of the chromosomally encoded AmpC enzymes (Helmy and Wasfi, 2014). AmpC β-lactamases mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor-β-lactam combinations. In many bacteria, AmpCs enzymes are inducible and can be expressed at high levels by mutation (Hall and Barlow, 2005).These enzymes

while they can be either chromosomally mediated or encoded by plasmids. Plasmid mediated AmpC enzymes are expressed at high levels hence, contribute significantly to clinical β-lactam resistance. Moreover, clinical isolates possessing plasmid encoding AmpC enzymes often are resistant to multiple antibiotics hence, leaving few therapeutic options (Barua *et al*., 2013) .

1.2 Rationale

Urinary tract infections (UTIs) are the most common outpatient infections. With the widespread use of β-lactam antibiotics, infections caused by resistant strains of bacteria is increasingly prevalent. One of the most important Beta-lactamse is AmpC, The increased presence of AmpC Beta-lactamase worldwide is becoming of great concern and infections caused by AmpC-positive bacteria are of particular clinical and epidemiological importance and cause higher patient morbidity and mortality (Helmy and Wasfi, 2014). Despite their importance in treatment failure specially with second and third generation cephalosporins. In sudan Few researches have been done to highlight the prevalence of AmpC enzymes among clinically isolated bacterial species compared to others enzymes.

1.3 . Objectives

1.3.1. General objective

To detect AmpC enzyme in gram-negative bacteria isolated from of patients with UTI .

1.3.2. Specific objectives

1.To isolate and identify gram-negative bacteria from patient with signs and symptoms of urinary tract infections.

2. To determine the antibacterial susceptibility of the isolated bacteria using Kirby-Bauer disc diffusion technique.

3. To detect the presence of AmpC enzyme in the isolated bacteria using modified three dimension method.

CHAPTER TWO

LITREATURE REVIEW

CHAPTER TWO

2-LITREATURE REVIEW

2.1. Background

Urinary tract infection (UTI) exists when bacteria adhere, multiply, and persist in a portion of the urinary tract. UTI causes vascular damage to the urinary bladder and decrease the competence of the kidney's functions, with subsequent conflicts in protein, acid–base, water and solute homeostasis and in the excretion of metabolic end products (El-Deeb and Buczinski, 2015).

This infections represent the most frequently reported community-acquired infection and carry a significant burden for patients' quality of life and healthcare costs (Cai *et al.*, 2018). It is estimated to affect 150 million people each year worldwide, with an annual incidence of 12.6% in women and 3% in men. Although most UTIs can be effectively treated by antibiotics UTI recurrence is a common problem and sometimes may be very troublesome (Jhang and Kuo, 2017).

The antimicrobial agents most commonly used to treat uncomplicated urinary tract infections include the combination drug trimethoprim and sulfamethoxazole, trimethoprim, β-lactams, fluoroquinolones, nitrofurantoin, and fosfomycintro-methamine (Jancel and Dudas, 2002). Genes expressing resistance to antibiotic have emerged in strains of bacteria and have disseminated through the global ecosystem to reach infecting microorganisms, produce disease, and seriously interfere with therapy, allowing infections to progress and kill despite antibiotic administration. The upsurge in prevalence of such resistance genes in the bacterial population that colonize and infect humans involves two processes, emergence and dissemination, in both of which there have been contributions from the developing world (Istúriz and Carbon, 2000).

It was identified that the inadequate access to effective drugs, the unregulated manufacture and dispensation of antimicrobials, and the lack of money available to pay for appropriate, high-quality medications are some of the major poverty-driven factors contributing to antimicrobial resistance in developing countries (Planta, 2007).

The causes of antimicrobial resistance (AMR) in developing countries are complex and may be rooted in practices of health care professionals and patients' behavior towards the use of antimicrobials as well as supply chains of antimicrobials in the population (Ayukekbong *et al*., 2017) .

Antibiotic-resistant bacteria are reported as the greatest threaten to human health in the world. unmindful use of antibiotics contributes to the occurrence of antibiotic resistance genes and the evolution of antibiotic-resistant bacteria according to the discipline of genesis and evolution (Wei *et al.,* 2018). One of the ways the bacteria use to gain resistance to antibiotic is the production of Beta-lactamases enzyme against Beta–lactams antibiotic and it should be noted that the first β-lactamase was identified in *Escherichia Coli* prior even to the release of penicillin for use in medical practice. The four major groups of β-lactams penicillin, cephalosporins, monobactams and carbapenems β-lactam ring which can be hydrolysed by β-lactamases resulting in microbiologically ineffective compounds (Gupta, 2007).

One of those enzymes is AmpC B-lactamases with their ability to confer resistances to penicillins, cephalosporins, oxyiminocephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g., cefoxitin) and monobactams and not be affected by the ESBL inhibitor (Ghonaim and Moaety, 2018).

2.1.1. Classification of Beta-lactamases

The most widely used classification of β-lactamases 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 structurally different metallo-β-lactamases that require a bivalent metal ion, usually Zinc, for activity. Later a new class of serine β-lactamases was found that bore little sequence similarity to the then-known class A enzymes. Designated class C, its members are also known as the 'AmpC' β-lactamases. Another class of serine β-lactamases, familiarly known as the OXA β-lactamases, was found to bear little resemblance to either class A or class C and was designated class D (Hall and Barlow, 2005).

The expanded version of the functional classification scheme proposed initially by Bush in 1989 and expanded in 1995. Enzymes were aligned based on their ability to hydrolyze specific β-lactam classes and on the inactivation properties of the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam (Bush and Jacoby, 2010).

2.1.2.1. Group 1 enzymes

These are Cephalosporinases belonging to molecular class C that are encoded on the chromosomes of many Enterobacteriaceae and a few other organisms (Bush and Jacoby, 2010).

2.1.2.2. Group 2 enzymes

Are serine β–lactamases include molecular classes A and D, represent the largest group of β-lactamases, due primarily to the increasing identification of ESBLs during the past 20 years. Subgroup 2a penicillinases represent a small group of β-

lactamases with a relatively limited spectrum of hydrolytic activity and are the predominant β-lactamases in Gram-positive cocci , including the *Staphylococci* and occasionally *Enterococci.* These enzymes preferentially hydrolyze benzylpenicillin and many penicillin derivatives, with poor hydrolysis of cephalosporins, carbapenems or monobactams and they are inhibited by clavulanic acid and tazobactam. Subgroup 2b β-lactamases readily hydrolyze penicillins and early cephalosporins, such as cephaloridine and cephalothin, and are strongly inhibited by clavulanic acid and tazobactam. They include the TEM-1, TEM-2, and SHV-1 enzymes, the most common plasmid-mediated β-lactamases identified in the 1970s and early 1980s. Subgroup 2be comprises ESBLs These broad-spectrum enzymes retain the activity against penicillins and cephalosporins of subgroup 2b β-lactamases and in addition hydrolyze one or more oxyimino-β-lactams, such as cefotaxime, ceftazidime, and aztreonam (Bush and Jacoby , 2010). ESBLs are most common in *Klebsiella pneumoniae* and *Escherichia coli*, but do occur in other Enterobacteriaceae and in *Pseudomonas aeruginosa*. ESBLs can be grouped into three main types: TEM, SHV and CTX-M (Heffernan *et al.,* 2007).

2.1.2.3. Group 3 beta-lactamases

Known as Metallo-β-lactamases (MBLs), a unique group of β-lactamases both structurally and functionally, are usually produced in combination with a second or third β-lactamase in clinical isolates. They differ structurally from the others β-lactamases by their requirement for a zinc ion at the active site. Functionally, they were once distinguished primarily by their ability to hydrolyze carbapenems, but some serine β-lactamases have the same ability. In contrast to the serine β-lactamases, the MBLs have poor affinity or hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam. Instead, they are inhibited by metal ion chelators such as EDTA and dipicolinic acid. (Bush and Jacoby.

2010). These Ambler class B enzymes have been isolated from *Pseudomonas* spp., *Acinetobacter* spp. and Enterobacteriaceae strains. They generally hydrolyse third-generation cephalosporins as well as carbapenems, but not aztreonam. The level of carbapenem resistance caused by MBLs is generally higher in Pseudomonas spp. than in Enterobacteriaceae, which often appear sensitive to imipenem (Drieux *et al*,. 2008).

2.1.2.3. AmpC Beta-lacatamases

AmpC β-lactamases are class C or group I cephalosporinases that confer resistance to a wide variety of β-lactam antibiotics including alpha methoxy β-lactams such as cefoxitin, narrow and broad spectrum cephalosporins, aztreonam, and are poorly inhibited by β- lactamase inhibitors such as clavulanic acid (Parveen *et al*., 2010).

Historicaly AmpC β-lactamases, demonstrated or presumed to be chromosomally mediated, have been described in *Acinetobacter* spp., *Aeromonas* spp., *Chromobacterium violaceum*, *C. freundii*, *Enterobacter* spp., *E. coli, Hafnia alvei*, *Lysobacter lactamgenus*, *Morganell amorganii*, *Ochrobactrum anthropi*, *Proteus rettgeri*, *Providencia stuartii*, *P. aeruginosa*, *Psychrobacter immobilis*, *Rhodobacter sphaeroides*, *S. marcescens*, and *Yersinia enterocolitica*. In many genera, AmpC is inducible via a system involving *ampD, ampG, ampR,* and intermediates in peptidoglycan recycling .The AmpC gene of *E. coli* is normally expressed at a low level, regulated by a growth rate-dependent attenuation mechanism but not by induction, since AmpR is missing while in *Shigella flexneri* and *Shigella dysenteriae, ampC* is included in a large deletion . An AmpC locus appears on the genetic map of *Salmonella* . but the evidence for its existence was indirect and its presence has not been confirmed in the sequenced genomes of *Salmonella enterica* serotypes Typhimurium or Paratyphi, so that *Salmonella* is

considered to be AmpC⁻. A chromosomal AmpC gene is also lacking in *Klebsiella* spp. and *P. mirabilis* (Philippon *et al.*, 2002). In most genera of the family *Enterobacteriaceae*, AmpC is induci*ble.* Unlike chromosome-mediated AmpC, plasmid-encoded AmpC enzymes are almost always expressed constitutively. Many plasmid-mediated AmpC enzymes, such as CMY-type β-lactamases, have been found in bacterial species that naturally lack a chromosomal AmpC β-lactamase, such as *Klebsiella pneumoniae, Proteus mirabilis, and Salmonella* spp. It is believed that such β-lactamases arose through the transfer of chromosomal AmpC genes onto plasmids (Parveen *et al*., 2010) before a plasmid-encoded ampC genes were thought to be non-inducible due to lack of a functional AmpR or absence of an AmpR binding site. However, two inducible plasmid-encoded Amp*C* genes of *M. morganii* origin, bla_{DHA-1} and $bla_{DHA-2,}$ have been described and Another example of plasmid mediated inducible AmpC will be the plasmid-encoded AmpC gene, bla_{ACT-1}, discovered in a *Klebsiella pneumoniae* isolate expressing at least five different β-lactamases, is inducible .This is the first inducible plasmid-encoded AmpC gene of Enterobacter origin to be described, thus destroying the generalization (Reisbig and Hanson , 2002). but mostly the detection of an AmpC β-lactamase *in Klebsiella* sp.*, Citrobacter koseri or Proteus mirabilis* is confirmatory for plasmid-mediated AmpC production because these organisms lack a chromosomal AmpC β-lactamases (Gupta *et al*., 2014). It is unnecessary to detect AmpC production in organisms that produce an inducible chromosomal AmpC β-lactamase because the organism identification is indicative of AmpC production; i.e., 100% of *E. cloacae, E. aerogenes, C. freundii, S. marcescens, Providencia sp., Morganella morganii, Hafnia alvei, Aeromonas spp.,* and *P. aeruginosa* isolates can be assumed to be AmpC producers (Thomson, 2010).

2.2.Epidemiology

AmpC b-lactamase AmpC producing Enterobacteriaceae can confer resistance to multiple antibiotics, such as penicillins, oxyiminocephalosporins like cefotaxime (CTX), ceftazidime (CAZ) and ceftriaxone and 7-a-methoxycephalosporins like cefoxitin and cefotetan (Teethaisong *et al*., 2016) .AmpC cephalosporinases are derivatives of the chromosomally encoded AmpC *β*-lactamases of bacteria such as *Enterobacter*, C. *freundii*, *M. morganii*, *Aeromonas* spp. and *Hafnia alvei* while most non inducible Ampc enzymes are plasmid mediated surveys of resistance mechanisms in cephamycin-resistant *E. coli* have identified a promoter or attenuator mutation, which results in the upregulation of naturally occurring chromosomal AmpC β-lactamase production and Occasionally, cephamycin-resistant *E. coli* can also produce plasmid-mediated AmpC β-lactamases (Pitout *et al*., 2010), while *K. pneumoniae* does not possess chromosomal AmpC. Therefore, detection of plasmid-mediated Amp*C* in *K. pneumoniae* is straightforward (Pérez-Pérez and Hanson, 2002).

In study done in Zurich, Switzerland by Peter-Getzlaff *et al* in 2011 to detect AmpC Beta-Lactamase in *Escherichia coli* in 51 clinical *E. coli* isolates with reduced susceptibility to amoxicillin-clavulanic acid, piperacillin-tazobactam, or extended-spectrum cephalosporins. 21/51 (41%) *E. coli* isolates were considered true AmpC producers. AmpC activity due to chromosomal AmpC promoter/attenuator mutations was found in 12/21 strains, and plasmid-carried AmpC genes were detected in 8/21 isolates. One strain contained both *AmpC* promoter mutations and a plasmid-carried AmpC gene (Peter-Getzlaff *et al*., 2011). While in another study done in New Zealandon by Barua *et al* in 2013, 250 *E. coli*, 199 *K. pneumoniae* and 51 of *K. oxytoca* isolates screened of AmpC production (showing an inhibition zone diameter of <18 mm against cefixitin

30ug). Of these 71 screen positive isolates of *E. coli*, 29 (40.8%) were identified as AmpC producers by modified three dimensional test, AmpC disk test as well as with inhibitor based detection method. However, AmpC disk test with Tris-EDTA could identify 19/71 (26.7%) isolates of *E. coli* to be AmpC producers. On the other hand 22/64 (34.3%) isolates of *K. pneumoniae* and 2/11 (18.1%) of *K. oxytoca*, were found to be AmpC producers by modified three dimensional test and AmpC disk test whereas, inhibitor based detection method identified 23/64 (35.9%) of *K. pneumoniea* and 2/11 (18.1%) of *K. oxytoca* to be AmpC producers. However, in comparison to these tests AmpC disk test with Tris-EDTA detected 20/64 (31.2%) *of K. pneumoniae* and 1/11 (9%) isolate of *K. oxytoca*to be AmpC positive (Barua *et al*., 2013) .

In study done to determine the occurrence of chromosomal and plasmid-mediated β-lactamases (AmpC) genes in a collection of Malaysian isolates of *Enterobacter* species using several phenotypic tests for detection of AmpC production of *Enterobacter sp* for 117 *Enterobacter* isolates revealed that 111 (94.9%) isolates as potential AmpC β-lactamase producers, 56 (50.5%) isolates were positive by the AmpC induction test (Mohd Khari *et al*., 2016).

Another study was done in Nigeria by Chika *et al* in 2015 to detect AmpC B-Lactamase among Anal *Pseudomonas aeruginosa* Isolates in a Nigerian Abattoir from a total of 25 *P. aeruginosa*. the isolates were highly resistant to ceftriaxone, ceftazidime, cefoxitin, gentamicin, ampicillin, sulphamethoxazole-trimethoprim and cefepime. while Ertapenem, nitrofurantoin and aztreonam exhibited antimicrobial activity against the *P. aeruginosa* isolates. 9 (36 %) *P. aeruginosa* isolates were phenotypically confirmed to produce AmpC beta-lactamase by the disk approximation method (Chika *et al*., 2015).

In a study done in Singapore by tan *et al* in 2008 investigating the prevalence of plasmid-mediated AmpC production in selected clinical isolates of *Escherichia coli*, *Klebsiella* species and *Proteus mirabilis* using *E. coli*, *Klebsiella* species and *P. mirabilis* with reduced susceptibility to amoxycillin-clavulanate, cefuroxime and cephalexin, but without phenotypic evidence of extended-spectrum β-lactamases plasmid mediated *AmpC* was present in 26% of study isolates (Tan *et al*., 2008).

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

3-MATERIALS AND METHODS

3.1. Type of study

The present study is descriptive cross-sectional study.

3.2. Study area

The study was carried out in El-Obaied Teaching Hospital. The experimental work was done in El-Obaied Specialized hospital and (SUST) Sudan University of Science and Technology Research Laboratory**.**

3.3.Study duration

The study was conducted during the period from Jun 2018 to February 2022

3.4 .Study population

Patient diagnosed with UTIs attending El-Obaied Teaching Hospital**.**

3.5. Ethical consideration

Participants in the study were informed and had being informed about this study aims prior to participation. All ethical rules were followed during sampling and data collection .permission to conduct this study was issued by the College of Medical Laboratory Science Ethical Committee (SUST).

3.6. Data analysis

Data were analyzed using SPSS version 16.

3.7. exclusion criteria

Specimens from Patient taking antibiotic within one week prior to date of collection were rejected.

3.8. Collection of sociodemographic data

Information regarding age, gender, educational level and antibiotic intake in the last three months were collected using pre designed form. (appendix I)

3.9. Collection of specimens

Urine specimens were collected from patient with signs and symptoms of UTI in clear sterile ,transparent, wide mouthed container. Patients were instructed to collect clear catch mid stream urine.

3.10. Transportation of specimens

All collected specimens were hold in $4^{o}C$ in refrigerator and transported within 2 hours to the Laboratory.

3.11. Isolation of bacteria

Urine specimens were inoculated on Cystine lactose Electrolyte Deficient medium (CLED) and incubated at $37^{o}C$ for 24 hours. Using a calibrated wire loop (freshly collected clean-catch specimen) by rotating the container. Using a sterile calibrated wire loop, (0.002 ml or 1/500 ml) one loopful was inoculated on plate of CLED agar and incubated aerobically at 37 °C overnight a colony forming unit of more than 10^5 was considered positive for urine culture (Cheesbrough, 2006).

3.12. Identification of bacterial isolate

3.12.1. Colonial morphology

The colonial morphology (color, size and shape) for each isolate was examined and reported.

3.12.2. Gram`s stain

The Gram stain was used to determine the gram reaction of the isolate, cell shape and arrangement were also determined. the method was performed as follows (Washington *et al*., 2006). A thin smear was prepared on a clean slide and fixed by rapidly passing the slide over the Bunsen flame. then the smear was covered with crystal violet and left for 1 minute. Then rinsed carefully with water .it was then covered with lugol's iodine and left for 1 minute afterword it was rinsed with water, decolorized with alcohol and rinsed again. The smear was covered with safranin for 2 minutes, rinsed and dried by blotting on a filter paper and examined under microscope by oil immersion lens, Gram- positive appear as violet color, while Gram-negative bacteria as red color.

3.12.3. Biochemical tests

3.12.3.1.Fermentation of sugars and production of gas and H_2S

Kliger iron agar (KIA) was used to determine if bacteria is able to ferment glucose and/or lactose and if the organism can produce hydrogen sulfide or other gases. These characteristics are used to identify various Enterobacteriacae. The presence of yellow color in both slope and butt means fermentation of glucose and lactose . if the color in slope is red and yellow in the butt that means fermentation of glucose only. The presence of black color mean H_2S is produced while air bubbles or the cracking or gab formation means gas production. The tube of KIA medium

was inoculated with test organism using sterile straight wire. multiple colonies were taken by wire loop and were stabbed into the agar butt (bottom of the tube) and then the slant was done in a wavy pattern. Results were read at 18-24 hours of incubation (betty *et al*., 2007).

3.12.3.2 Citrate utilization test

This test used to detect the ability of an organism to utilize citrate as the sole source of carbon for energy. Bacteria were inoculated on a medium containing sodium citrate and a pH indicator Bromothymol blue. The medium also contains inorganic ammonium salt, which is utilized as a sole soruce of nitrogen . utilization of citrate involves the enzyme citratase, which breaks down citrate to oxaloacetate . Oxaloactate will further be broken down to pyruvate, CO_2 and the production of $NaCO_3$ as well as NH3 from utilization of sodium citrate and ammonium salt respectively resulting in alkaline pH. This results in change of medium colour from green to blue. Suspected colonies were picked up with sterile straight wire and inoculated into slope of simmons's citrate agar and incubated overnight at 37^{o} C. if the organism had the ability to utilize citrate, the medium changes its colour from green to blue (PHE, 2014) .

3.12.3.3. Urease test

This test demonstrates the ability of bacterium to produce the enzyme urease , which is capable of hydrolyzing urea changing the pH of the medium. Urea agar was inoculated with the organism under test and incubated for 24 hours at 37^{o}C, if the colour changes to magenta that means positive result (Betty *et al*., 2007).

3.12.3.4. Indole test

Some bacteria can produce indole from the degradation of amino acid tryptophan using the enzyme tryptophanase to produce indole, ammonium and pyruvate. Production of indole is detected using Kovac`s reagent, indole react with the aldehyde in the reagent to give a red colour. An alcoholic layer concentrate the red colour as a ring at the top.

The test was performed by inoculating bacteria under test into peptone water, containing tryptophane amino acid and incubated overnight at 37^{o}C. Following incubation period a few drops of kovac`s reagent that contain dimethyl aminobenzaldehyde (DMAB) The Formtion of a red or pink coloured ring at the top is taken as positive (PHE, 2014).

3.12.3.5 Motility test

Motility in bacteria can be provided by a variety of mechanisms, but the most common involve flagella ,The presence of flagella occurs primarily in bacilli but there are a few flagellated cocci, thus motility is a very important means of identification in the family Enterobacteriaceae, motility test medium is used to determine the motility of microorganisms (Shields and Cathcart, 2011).

The motility test tube medium were inoculated using the stab technique until two third of the medium and then incubated at 37^{o}C for 24 hours, afterwords the test tubes were examined for the presence or absence of growth along the line of the stab , with cloudiness of the medium caused by Organisms migration from the stab line and diffusing into the medium, which appear as turbidity is considered positive result. And a visible stab line and clear agar media or Growth accentuated along the stab line but no further and surrounding medium remains clear is considered negative result (PHE, 2016).

3.13. Antimicrobial susceptibility testing

Kirby-Bauer disc diffusion technique was done using (Oxoid Antimicrobial Susceptibility Test Discs), against Imipenem (10mcg), Ciprofloxacin (5mcg), Gentamicin (10mcg), Ceftazidime (30mcg), Nitrofourantoin (300mcg) and Co-trimoxazole (25mcg). A disc of blotting paper is impregnated with a known volume and appropriate concentration of an antimicrobial, and this is placed on a plate of susceptibility testing agar uniformly inoculated with the test organism. The antimicrobial diffuses from the disc into the medium and the growth of the test organism is inhibited at a distance from the disc that is related (among other factors) to the susceptibility of the organism. a sterile dry surfaced Mueller Hinton agar medium with 4mm depth prepared in level surface was used and microorganism incoulum is prepared against Mcfarland standard 0.5 in sterile physiological saline and turbidity was matched against good light source. Using a sterile swabs Mueller Hinton agar media were inoculated the swab was Stroked evenly over the surface of the medium in three directions, rotating the plate approximately 60% to ensure even distribution and petri-dish lid in place for no longer than 15 minutes for surface of the agar to dry. A sterile forceps was used to place the antimicrobial discs on the inoculated plate . the Interpretative Chart was used and the zones sizes of each antimicrobial were reported as 'Resistant', 'Intermediate/Moderately susceptible' and 'Susceptible'. after an overnight incubation the zones diameter (including the disk) were measured with ruler on the undersurface of the petri-dish .(Bauer *et al*., 1996) and (Cheesbrough, 2006) .

3.14. AmpC enzyme detection using Modified three dimension test

A lawn of *E. coli* ATCC 25922 was made on MHA plate, 30µg cefoxitin (FOX) disk was placed in the center. Linear slits (3 cm) was made radially, 5 mm away from the edge of cefoxitin (FOX) disk.5-10 colonies of the test organism were inoculated with the help of nichrome loop in the slit, beginning near the disc and moving outwards. After overnight incubation, the plates were observed for zone distortion as AmpC positive or no zone distortion as AmpC negative, *Escherchia coli* ATCC 25922 was used as control negative (Thakar and Modak, 2013).

CHAPTER FOUR

RESULTS

CHAPTER FOUR

4-RESULTS

A total of 100 urine specimens were collected from patients with signs and symptoms of urinary tract infection (13 males and 87 females) attending El-Obied Teaching Hospital .

Sixty two (n=62) bacteria were isolated. 10(16.1%) were gram positive and 52 (83.9%) were Gram negative bacteria which were 15(24.2%) *Escherichia coli*, 9(14.5) *Enterobacter aerogenes* , (9) *Proteus mirabilis*, (8) *Klebsiella pneumoniae*, (4) *Pseudomonas aeruginosa*, (3) *Citrobacter freundii*, (3) *Serratia marecens* and (1) *Protues vulgarius* (table 3-1). Identification was done by colonial morphology on Cystine Lactose Electrolyte Deficient medium and blood agar, Gram`s stain technique to differentiate between gram positive and gram negative bacteria. fermentation of sugars and production of gas and H_2S, Urease test, Indole test and Motility test for identification of gram negative bacterial(table 3-2).

Antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion technique. The antibiotic susceptibility was done to test for Imipenem (10mcg), Gentamicin (10mcg), Ciprofloxacin (5mcg), Ceftazidime (30mcg), Nitrofourantoin (300mcg) and Co-trimoxazole (25mcg). (Tables 3-3,3-4). AmpC detection was done by the non extract based modified three dimension method. Of the 52 Gram negative isolates (46.2%) were AmpC positive and (53.8%) were negative, (Table3-5).

Antimicrobial susceptibility showed that 22/35 of ceftazidime resistant isolates are AmpC positive, while 4/6 of Ciprofloxacin resistant, 2/2 of gentamicin resistant, 7/20 nitrofurantoin resistant , 11/21 of Co-trimoxazole resistant, and 2/4 of Imipenem resistant isolates were AmpC positive, (Table 3-6).

Of the 52 isolates tested for AmpC enzyme, 5(9.6%) were from male patients and 47(90.4%) were from female patients, (Table 3-7). and form age groups that range from 5 years to 65 years. The mostly encountered age groups were from 26 to 45 years from which most isolate were obtained(Table3-7). Among isolates tested for AmpC it was noted that 30 (57.6%) of the patients only have basic level of education (can read and write) and (11.5%) can`t at all, showing prevalence of AmpC of 13/30 and 4/6 respectively,(figure 3-1).

We report 14 (58.8%) of the 24 AmpC positive isolate were from patients whom have taken antibiotic drugs within the last three months, (figure 3-2).

table (3-1). frequency of isolates

bacteria	Frequency	Percent
Gram-positive	10	16.1
Escherichia coli	15	24.2
Enterobacter aerogenes	9	14.5
Klebsiella pneumoniae	8	12.9
Pseudomonas aeruginosa	4	6.5
Citrobacter freundii	3	4.8
Proteus mirabilis	9	14.5
Protues vulgarius	1	1.6
Serratia marecens	3	4.8
Total	62	100.0

Table 3-2. Entity, frequency and Biochemical identification of Gram`s negative isolates form urine specimens

BIOCHEMICL TEST for identification of the isolate							ORGANISM	Frequency
Glucose ferment-ation	Lactose fermenta-tion	Gas and H_2S	Citrate utilization	Urease productin	Indole test	Motility test		
+	+	gas	-	-	+	+	*E. coli*	15
+	+	gas	+	-	-	+	*Enterobacter aerogenes*	9
+	+	gas	+	+	-	-	*K .pneumoniae*	8
-	-	-	+	-	-	+	*P. aeruginosa*	4
+	+	*gas*	+	*d*	-	+	*C. freundii*	3
+	-	*gas/H_2S*	+	+	-	+	*P. mirabilis*	9
+	-	*gas(d)* *H_2S*	*d*	+	+	+	*P. vulgarius*	1
+	*D*	*gas(d)*	-	*d*	-	+	*S. marecesns*	3

Key words: +: positive, - :negative, d: doubtful

Table 3-3. Antibiotic Susceptibility results.

Bacteria	Imipenem			Gentamicin			Ciprofloxacin		
	S	I	R	S	I	R	S	I	R
Escherichia coli	9	6	0	15	0	0	14	0	1
Enterobacter aerogenes	5	3	1	6	2	1	9	0	0
K. pneumoniae	4	3	1	6	2	0	6	1	1
P. areuginosa	3	1	0	4	0	0	3	0	1
C. freundii	3	0	0	1	1	1	2	0	1
Proteus mirabilis	5	3	1	9	0	0	9	0	0
Proteus vulgarius	0	0	1	0	1	0	0	0	1
Serratia marecesns	3	0	0	3	0	0	2	0	1
Total	32 61.6%	16 30.7%	4 6.7%	44 84.6%	6 11.6%	2 3.8%	45 86.5%	1 1.9%	6 11.6%

Keys: R :Resistance, S : Susceptible, I :Intermediate resistance

Table 3-4. Antibiotic susceptibility results.

Bacteria	Ceftazidime			Nitrofourantoin			Co-trimoxazole		
	S	I	R	S	I	R	S	I	R
Escherichia coli	2	4	9	12	0	3	8	0	7
Enterobacter aerogenes	2	0	7	6	1	2	5	0	4
K.pneumoniae	1	2	5	3	0	5	3	1	4
P.areuginosa	0	1	3	2	0	2	2	1	1
C. freundii	0	1	2	3	0	0	1	0	2
Proteus mirabilis	2	2	5	3	0	6	8	1	0
Proteus vulgarius	0	0	1	1	0	0	0	0	1
Serratia marecesns	0	0	3	1	0	2	1	0	2
Total	7 13.5%	10 19.2%	35 67.3%	31 59.6%	1 1.9%	20 38.5%	28 53.8%	3 5.8%	21 40.4%

Keys: R : Resistance, S : Susceptible, I :intermediate Resistance

Table 3-5. Distribution of AmpC positive isolate according to species.

	AmpC		Total
	positive	negative	
Escherichia coli	4(7.7%)	11(21.1%)	15(28.8%)
Enterobacter spp	7(13.4%)	2(3.9%)	9(17.3%)
Klebsiella pneumoniae	2(3.9%)	6(11.5%)	8(15.4%)
Pseudomonas aerugionsa	4(7.7%)	0(0%)	4(7.7%)
Citrobacter furendii	2(3.9%)	1(1.9%)	3(5.8%)
Proteus mirabilis	1(1.9%)	8(15.4%)	9(17.3%)
Proteus vulgaris	1(1.9%)	0(0%)	1(1.9%)
Serratia marecesns	3(5.8%)	0(0%)	3(5.8%)
Total	24(46.2%)	28(53.8%)	52(100%)

Table 3-6. Antibiotic Susceptibility results as Susceptible, Intermediate and Resistance for AmpC positive and negative isolates.

		Ceft	Nitro	Cipro	Genta	Co-tri	Imipenem
AmpC Positive N= 24	Susceptible	0 0%	16 66.7%	20 83.3%	16 66.7%	11 45.8%	16 66.7%
	Intermediate	2 8.3%	1 4%	0 0%	6 25%	2 8.3%	6 25%
	Resistance	22 91.7%	7 29.2%	4 16.7%	2 8.3%	11 45.8%	2 8.3%
AmpC Negative N= 28	Susceptible	7 25%	15 53.6%	25 89.3%	28 100%	17 60.7%	16 57.1%
	Intermediate	8 28.6%	0 0%	1 3.6%	0 0%	1 3.6%	10 35.7%
	Resistance	13 46.4%	13 46.4%	2 (7.1%)	0 0%	10 35.7%	2 7.1%

Keys: Ceft: Ceftazidime, Nitro: Nitrofurantoin, Cipro: Ciprofloxacin, Genta: Gentamicin, Co-tri: Cotrimoxazole

Table 3-7. Distribution of AmpC positive isolates among patients genders

	AmpC	positive	negative	Total
Sex	male	4(7.7%)	1(1.9%)	5(9.6%)
	female	20(38.5%)	27(51.9%)	47(90.4%)
Total		24(46.2%)	28(53.8%)	52(100%)

showing insignificant p value= 0.110.

Table 3-8. Distribution of AmpC positive isolates among age groups

	AmpC	positive	negative	Total
age	5-15	2(3.9%)	1(1.9%)	3(5.8%)
	16-25	4(7.7%)	3(5.8%)	7(13.5%)
	26-35	6(11.5%)	13(25%)	19(36.5%)
	36-45	9(17.4%)	6(11.4%)	15(28.8%)
	46-55	1(1.9%)	2(3.9%)	3(5.8%)
	56-65	2(3.8%)	3(5.8%)	5(9.6%)
Total		24(46.2%)	28(53.8%)	52(100%)

showing insiginificant p value = 0.563

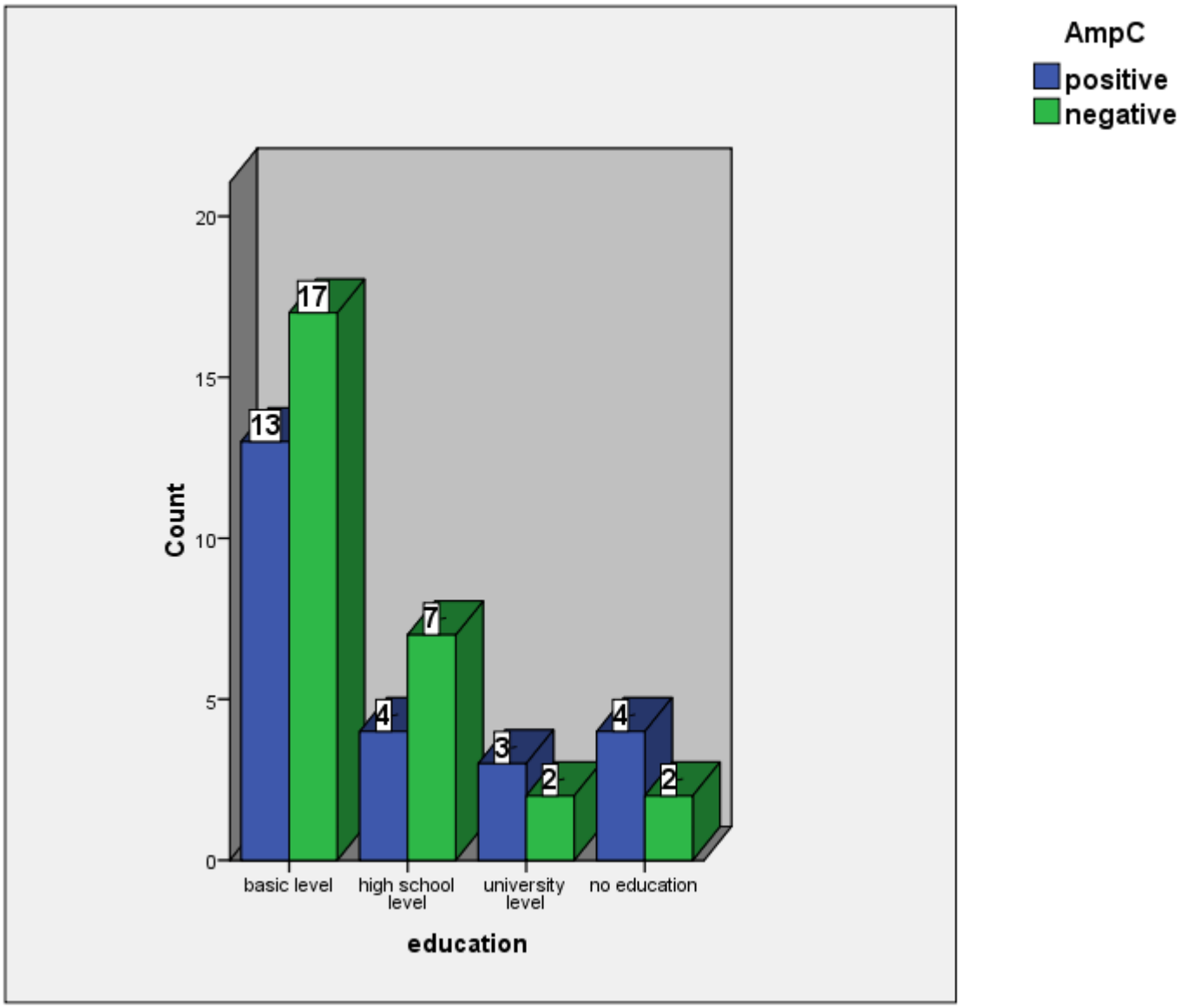

Figure (3-1): distribution of AmpC positive isolates among patient according to the level of education of patient showing insignificant p value =0.589

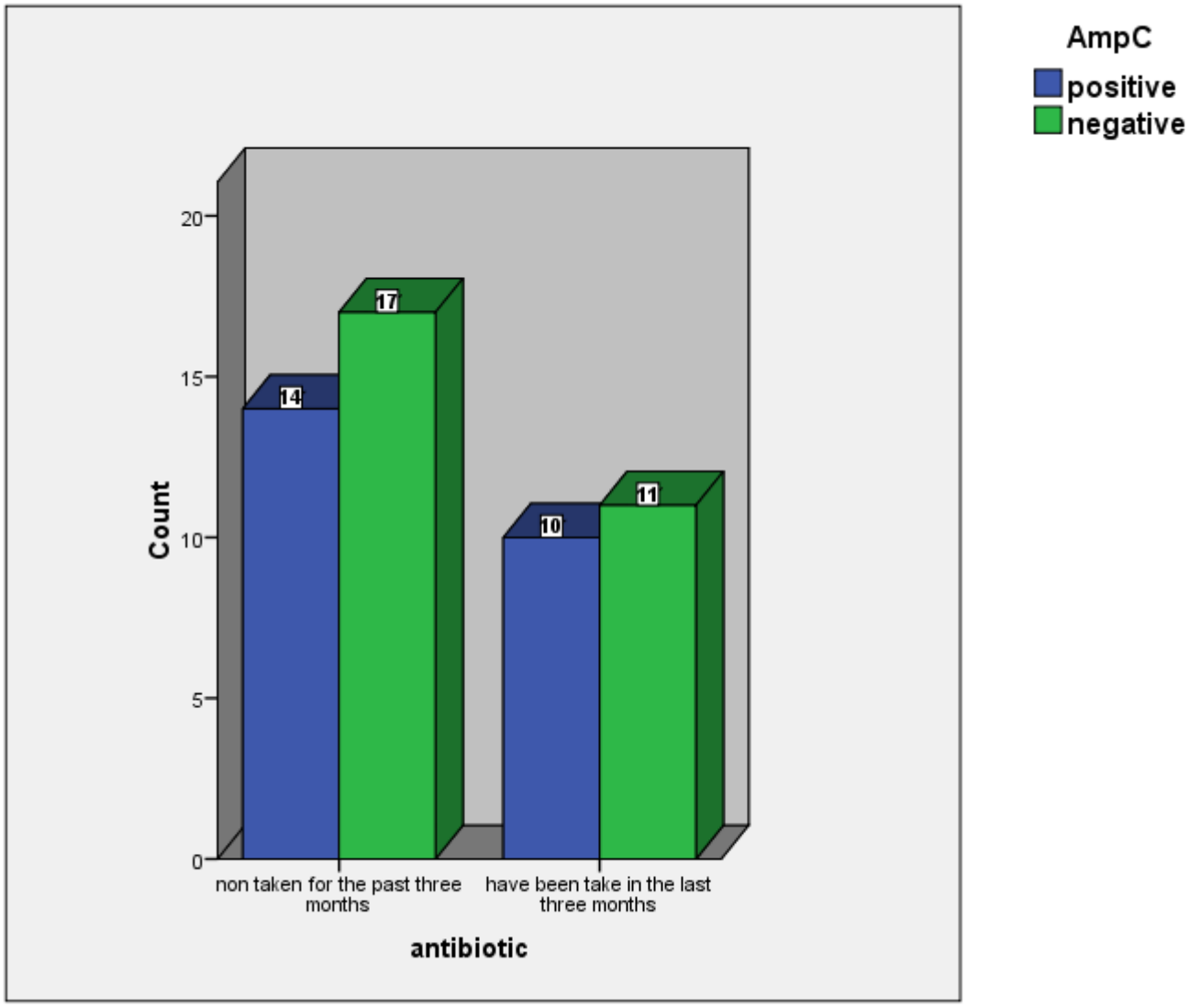

Figure (3-2), distribution of AmpC positive isolates among patient according to antibiotic intake within three months showing insignificant p value= 0.862

CHAPTER FIVE

DISCUSSION, CONCLOSION AND RECOMMENDATION

CHAPTER FIVE

5-DISCUSSION, CONCLOSION AND RECOMMENDATION

5.1. Discussion

Sixty two organisms were isolated, the obtained results were 10 (16.1%) Gram-positive and 52 (83,9%) Gram-negative as; 15 (24.1%) *Eschersicha coli* , 9 (14.5%) *Enterobacter aerogenes*, 8 (12.9%) *Klebsiella pneumoniae*, 9 (14.5%) *Proteus mirabilis* , 4 (6.5%) *Pseudomonas aeruginosa*, 3 (4.8%) *Citrobacter freundii*, 3 (4.8%) *Serratia marecens* and 1 (1.6%) *Protues vulgarius*. Our results are less than Saeed *et al*.,(2017) who isolated (35%) Gram-positive bacteria (as 19% *Enterococcus faecalis*, 13% *Staphylococcus aureus* and 3% *S saprophyticus*) and (54%) *E coli* (4%) *klebsiella pneumoniae*,(4%) *proteus mirabilis* (3%) *pseudomonas spp* (Saeed *et al*., 2017) and the results obtained by Seifu and Gebissa, (2018) who isolated (31.6%) gram positive bacteria (as 20.2% *Staphylococcus aureus* and 11.4% *lecuonostoc* species) , (39.3%) *Escherichia coli*, (8.4%) *klebsiella* spp and (5.2%) *Citrobacter fruendii* (Seiu and Gebissa, 2018) difference was attributed to epidemiological reasons.

We reported the susceptibility to Ciprofloxacin was the highest (86.5%) followed by Gentamicin (84.6%), Impenem (61.6%), Nitrofourantoin (59.6%) and Co-trimoxazole (53.8%), in contrast to Seifu and Gebissa, (2018) who reported 93.3%, of isolates as sensitive to gentamicin. Similarly, 60%, 56.6%, 46.6%, of the isolates were sensitive to nitrofurantoin, ciprofloxacin, Trimethprime-Sulfamethoxazole respectively (Seiu and Gebissa, 2018), difference is attributed to different ratio of isolates and epidemiology of resistance.

In this study AmpC was tested among 52 gram-negative organisms, 24(46.2%) were positive, and 28 were negative(53.8%) distributed as 4 (7.7%) *Escherichia*

coli, 7 (13.4%) *Enterobacter* aerogenes isolates, one (1.9%) *Proteus mirabilis* isolates, 2 (3.9%) *Klebsiella pneumoniae* isolates, all the 4(7.7%) *Pseudomonas aerugionsa* isolates, one (1.9%) *Citrobacter freundii, 3(5.8%) Serratia marecens isolates* and the *one proteus vulgaris*, were AmpC positive by M3D test, This result is more than the results obtained by Helmy and Wasif (2014) of (102) *Escherichia coli,* (30) *Klebsiella pneumoniae,* (5) *Klebsiella oxytoca,* (4) *Proteus mirabilis,* and (2) *Proteus vulgaris. Escherichia coli* resulted in 15.6% (16) AmpC positive and *K. pneumoniae* in 3% (only one organism) while others were negative (Helmy and Wasif, 2014), Polsfuss *et al.,* (2011) reported *one Proteus mirabilis* from 131 isolates (Polsfuss *et al.,* 2011) and the results obtained by Mwinga *et al*., (2018) which was (10.77%) of 130 isolated *E .coli* (Mwinga *et al*., 2018), while less than the results obtained by Handa *et al*., (2013) where AmpC enzyme in *E. coli* was detected in 39% of clinical isolates of *E. coli* using IBM and M3D methods respectively. While our results are similar to Gupta *et al*., (2012) who reported 32% (32 of 100) *Klebsiella pneumonaie*. Results reflect epidemiological differences (Handa *et al*., 2013)

We reported no significant relations between infection by AmpC producer organism and suspected risk factors; gender, age , educational level , recent antibiotic intake (p value > 0.5) while Zerr *et al*., (2016) reported that in general prior antibiotic intake does not hold a significant risk unless the patient have taken Third-genration cephalosporin (Zerr *et al.,* 2016).

5.2. Conculsion

Infections caused by AmpC producing strains are emerging. This study conclude that there was considerable ratio of UTIs due to AmpC producing Gram- negative bacteria in El-Obaied city (n=24) along with hight resistance to Ceftazidime, Nitrofurantoin and Cotrimoxazole.

5.3. Recommendation

Implementation of phenotypic detection methods of AmpC enzymes in routine laboratory testing to help in the development of effective treatment strategy along with genetic methods to help identify the epidemiology of those enzyme and to track from where those resistances enzymes emerge.

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APPENDEXES

Sudan University of Science and Technology

College of Graduate Studies

Questionaree:

Sample NO:……

1. gender: Male……. Female ……

2.Age:…….Years

3.Have taken antibiotic in the last 3 months:

Yes…… NO…….

4.Level of education

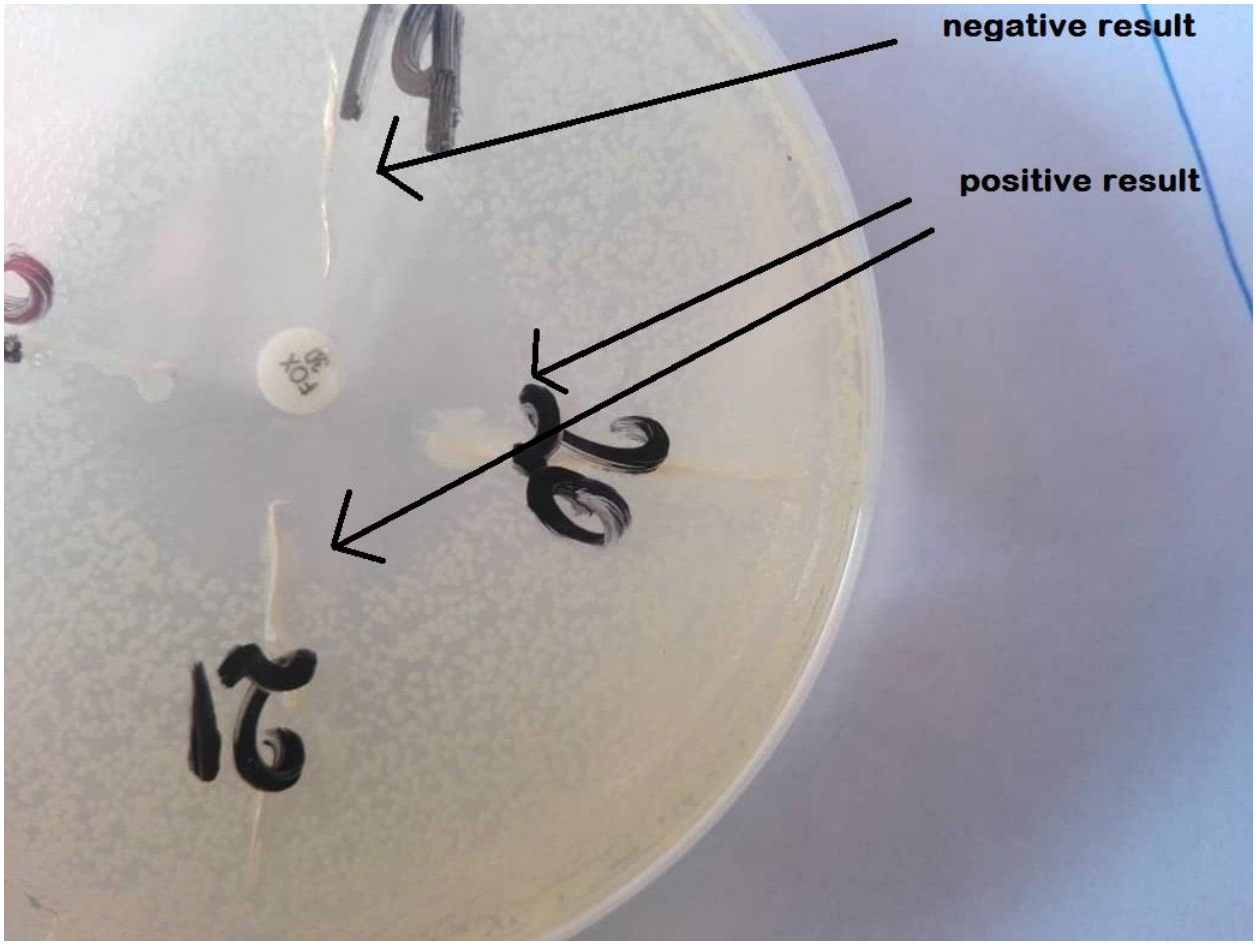
No education (Can't read and write)…….

Basic ……..

High school……..

University……

Appendix (1). Questionaree.



Appendix (2).Modified three dimension test for AmpC detection