CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Microorganisms have existed on the earth for more than 3.8 billion years and exhibit the greatest genetic and metabolic diversity. In order to survive, they have evolved mechanisms that enable them to respond to selective pressure exerted by various environments and competitive challenges (Byarugaba, 2010). The disease causing microorganisms have particularly been vulnerable to man's selfishness for survival who has sought to deprive them of their habitat using antimicrobial agents. These microorganisms have responded by developing resistance mechanisms to fight off this offensive (Kong *et al*., 2010).

Resistance of pathogenic organisms to countenance antibiotics has become a worldwide problem with serious consequences on the treatment of infectious diseases. The heightened use/misuse of antibiotics in human medicine is primarily contributing to the phenomenon (Alekshun and Levy, 2007). Beta-lactam antimicrobial agents exhibit the most common treatment for bacterial infections and continue to be the prominent cause of resistance to β-lactam antibiotics among Gram-negative bacteria worldwide (Zapun *et al*., 2007). Since discovery of penicillin 1921 by Alexander Fleming has provided a safe and effective treatment for multitude of infection (Kong *et al*., 2010). Cephalosporin has wide range of indication carbapenems are using in nosocomial infection and infection multi-resistance microorganism (Rasha and Hammad, 2016).These antibiotics work by inhibiting cell wall biosynthesis in the bacteria (Benton *et al*., 2007). The most widespread cause of resistance to these antibiotics is production of enzyme called β-lactamase which produced by many Gram-positive and Gram negative bacteria that inactivate these

antibiotics by opening the β-lactam ring (Lakshmi *et al*., 2014; Adam, 2002). The persistent exposure of bacterial strains to a multitude of β-lactams has induced dynamic and continuous production and mutation of β-lactamases in these bacteria, expanding their activity even against the newly developed β-lactam antibiotics (Paterson and Bonomo, 2005).

Within a few years, most hospital isolates had become resistant to penicillin and were found in patients who were previously treated with the drug. Till the early 1950s, penicillinase producing *Staphylococcus aureus (S.aureus)* strains were universally present in hospitals, whereas community isolates continued to depict susceptibility.Then, astudy conducted during 1957-1966 in Copenhagen showed that 85% - 90% of hospital strains and 65% - 70% of community strains were resistant to penicillin, demonstrating that resistance was an increasingly alarming phenomenon and a much more prevalent conundrum than what had been originally anticipated (Bidya and Suman, 2014). In a study for PhD, Ali compared antibiotic susceptibilities of Enterobacteriaceae isolated in Khartoum Teaching Hospital and Soba University Hospital. He found that resistance of *Escherichia coli* (*E.coli*) and *Klebsiella* spp. to ceftriaxone and ceftazidime in both hospitals ranged from 56.5% to 79% (Ali, 2013).

Several methods are available for detecting the production of penicillinase by bacteria includes: iodometric, acidimetric, chromogenic cephalosporin (Llanes *et al*., 2003; David and Brown 2005).

1.2. Rationale

The resistance to β-lactam antibiotics was recognized as an increasingly global problem.

Reports on antibiotics resistance from Sudan are scarce and little is known about the ratio of antimicrobial resistance due to ESBLs so the research in this area emphasizes the need for more enforcement.

1.3. Objectives

1.3.1. General objective

To detect extended spectrum βeta-lactamase among bacteria isolated from patients in El-obied Hospital.

1.3.2. Specific objectives

1. To isolate bacterial species from different clinical specimens
2. To perform antimicrobial susceptibility testing.
3. To detect ESBLs in isolated bacteria using Double Disk Synergy Test and Combined Disk Method.
4. To correlate between the presence of ESBLs and type of clinical specimens, gender, age, education and occupation.

CHAPTER TWO

LITERATURE REVIEW

2.1. Antibiotics

Antibiotics were discovered in the middle of the nineteenth century and brought down the threat of infectious diseases which had devastated the human race. However, soon after the discovery of penicillin in 1940 (Kong *et al*., 2010), a number of treatment failures and occurrence of some bacteria such as staphylococci which were no longer sensitive to penicillin started being noticed. This marked the beginning increasing prevalence of resistance has been reported in many pathogens over the years in different regions of the world including developing countries. This has been attributed to changing microbial characteristics, selective pressures of antimicrobial use, and societal and technological changes that enhance the development and transmission of drug-resistant organisms (Byarugaba, 2005).

Although antimicrobial resistance is a natural biological phenomenon, it often enhanced as a consequence of infectious agents' adaptation to exposure to antimicrobials used in humans or agriculture and the widespread use of disinfectants at the farm and the household levels (Walsh, 2000). It is now accepted that antimicrobial use is the single most important factor responsible for increased antimicrobial resistance. In general, the reasons for increasing resistance levels include the following: suboptimal use of antimicrobials for prophylaxis and treatment of infection prolonged hospitalization, increased number and duration of intensive- care-unit stays, increased use of invasive devices and catheters, transfer of colonized patients from hospital to hospital and antibiotic use in agriculture and household chores (Aarestrup *et al*., 2001).

2.1.1. Mechanisms of Action of Antimicrobial Agents

The mechanism of action of antimicrobial agents can be categorized further based on the structure of the bacteria or the function that is affected by the agents. These include generally the following: inhibition of the cell wall synthesis, inhibition of ribosome function, inhibition of nucleic acid synthesis (Kapoor *et al*., 2017).

2.1.2. Mechanisms of Antimicrobial Resistance

Antibiotics resistant are due to one of four general mechanisms). Like; inactivation of antibiotic by β-lactamase, modification of target penicillin-binding protein (PBPs), impaired penetration of drug to target PBPs efflux (Majiduddin and palzkill, 2002).

2.2. β-lactam antibiotic

Are class of broad spectrum antibiotic contain beta lactam ring in their molecular structure (Livermore *et al*., 2001). This includes penicillin derivatives (penams) cephalosporin (cephem) monobactam and carbapenems (Holten *et al*., 2000).

2.2.2. Mechanism of action of β-Lactma antibiotics

The cell wall of bacteria is composed of a complex cross linked polymer of polysaccharide and polypeptides like peptidoglycan. The polysaccharide contains alternating amino sugar, N-acetyl glucosamine and N-acetyl muramic acid sugar. This peptide terminates in D-alanyl-D-alanine. Penicillin- Binding Protein is an enzyme that removes the terminal alanine in process of forming a cross link with a nearby peptide. The β-lactams inhibit the final transpeptidation by forming covalent bond with penicillin-Binding Protein that has transpeptidase and carboxypeptidase activities thus preventing formation of the cross links.

The final bactericidal action is the inactivation of an inhibitor of autolytic enzymes in the cell wall, which leads to the lysis of the bacteria (Lakshmi *et al*, 2014).

2.2.2. β-lactamase

Is a plasmid-encoded enzyme that hydrolyzes β-lactam ring of β-lactam antibiotics rendering them ineffective. These enzymes, produced by *S. aureus* along with many other organisms, have hindered the use of many useful and once life-saving β-lactam antibiotics from clinical practice (Bidya and Suman, 2014).

2.2.3. Localization of β-lactamases

In Gram-negative bacteria, enveloped by their outer cell wall, the β-lactamases are strategically placed in the potential "peri-plasmic space" where compounds entering the cell via the porins would be immediately detected.

In Gram-positive bacteria that lack this outer cell wall, β-lactamases are similarly synthesized and secreted into a "cloud" surrounding the organism. Thus, antibiotics that approach the organism must be stable to these enzymes once they reach the vicinity of the bacteria (Hall, 2003).

2.2.4. Class of β-Lactare amases

About 300 β-lactmases are known. They broadly prevalent enzymes that are classified using main classification system: Ambler (structural) and bush-Jacoby-Mederiros (functional).

Ambler classification of β-lactamases

2.2.4.1. CLass A β-lactamases

2.2.4.1.1. Extended-Spectrum β-lactamase

Are mutant enzymes with wide range of activity than their parent molecules. They hydrolyze third and fourth generation cephalosporin and aztrenam but don't affect the second generation cephalosporin and remain β-lactamase inhibitors susceptible. The common plasmid-mediated β-lactamase in enterobacteriaceae are TEM-1, TEM2 and SHV-1(Jacoby and Munoz, 2005).

2.2.4.1.1.1. Plasmid-Encoded transposable Element β-lactamase (TEM)

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

2.2.4.1.1.2. SHV β-lactamase

SHV-1 shares 68 % of its amino acid with TEM and has a similar overall structure. SHV-1 β-lactamase is most commonly found in *Klebsiella pneumoniae* . More than 60 SHV varieties are known. SHV-5 and SHV-12 are among the most common (Paterson *et al*., 2003).

2.2.4.1.1.3. CTX-M β-lactamase

These enzymes were named for greater activity against cefotaxime than other oxyimino-beta-lactam substrates. These enzymes are not very

closely related to TEM or SHV β-lactamase in that they show only approximately 40% identity with these two commonly isolated β-lactamase. They have mainly been found in strain of *Salmonella enteric* serovar *typhimurium* and *E. coli* and other enterobacteriaceae (Hudson *et al*., 2014).

2.2.4.1.2.4. *K.pneumoniae* carbapenemase (KPC)

A few class A enzymes, most noted the plasmid-mediated KPC enzymes, are effective carbapenemasea as well. Ten variants, KPC-2 through KPC11 are known (Nordmann *et al*., 2009).

2.2.2.1.4. Class B

2.2.4.1.2.1. Metallo-β-lactamases

It is though still rare and predominantly reported from the Far East, acquired these require enzymes such as zinc or heavy metals for catalysis and their activity is inhibited by chelating agent. These classes of enzymes are resistant to inactivation by clavulanate, sulbactam, aztreonam and carbapenems (David and Brown 2005).

2.2.4.1.2.2. IMP-type crbapenemases

Plasmid mediated IMP-type crbapenemases, 19 varieties of which are currently known. Become established in Japan in enteric Gram-negative bacteria and in *Pseudomonas* and *Acinetobacter* species.IMP enzyme spread slowly to other countries in the far east (Makena *et al*., 2016).

2.2.4.1.2.3. Verona intrgron-encoded metallo-β-lactamases (VIM)

VIM family was reported from Italy in 1999 and now includes 10 members. VIM-1 was discovered in *Pseudomonas aeruginosa*, VIM-2 now the predominant variant-was found in Europe. VIM enzymes occur

mostly in *P. aeruginosa*, also *P. putida* and very rarely Enterobacteriaceae (Makena *et al*., 2016).

2.2.4.1.2.4. NDM-1(New Delhi metallo-β-lactamase

Originally described from NEW Delhi in 2009, this gene is now widespread in *E. coli* and *K. pneumoniae* from India and Pakistan.NDM have several variant which share different properties (Walsh *et al*., 2000).

2.2.4.1.3. Class C β-lactames

2.2.4.1.3.1. AmpCs

They are not inhibited by β-lactamase inhibitors. That are repressed and produced at low levels. Plasmid mediated AmpCs are also inducible. Two mechanisms responsible for AmpCs activity in *E. coli* are mutation in AmpCs promoter and attenuator regions resulting in AmpCs over expression and acquisition of plasmid-carried AmpCs genes (Philippon *et al*., 2002; Bradford, 2001).

2.2.4.1.3.2. CMY

The first class C carbapenemase was described in 2006 and was isolated from a virulent strain *of Enterobacter aerogenes*. It is carried on plasmid, PYMG-1, and is therefore transmissible to other bacterial strain (lee *et al*., 2004).

2.2.4.1.4. Class D β-lactamase

2.2.4.1.4.1. OXA β-lactamase

The OXA type enzymes are produced by enterobacteriaceae and *P.aerugenosa*. they pose resistance against amino and ureidopenicillin and high level hydrolytic activity against cloxacillin, oxacillin and methicillin while most EBSL have been found in *E. coli*, *K. pneumoniae*,

P. aeruginosa more than 80 CTX-M enzyme are known CTX-M-14, CTX-M-3 and CTX-M-2 are most widespread (David and Brown, 2005).

2.2.5. β.-lactamase inhibitors

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

2.2.6. Tests for detection of β-lactamases

2.2.6.1. Acidimetric method

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

2.2.6.2. Iodometric method

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

2.2.6.3.Nitrocefintest.

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

amounts of the nitrocefin solution are added. β-Lactamase activity is indicated by a red colour within 1-2 minute (David and Brown, 2005).

2.2.6.4. Microbiological tests of β-lactamase activity

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

2.2.6.4.1. Modified Hodge test (MHT)

Is recommended by Clinical and Laboratory Standards Institute as a confirmatory test for carbapenemase production. The MHT was performed by preparing 0.5 McFarland dilution of Escherichia coli ATCC 25922 in 5 ml saline. Then 1:10 dilution of this preparation is streaked as a lawn on a Mueller Hinton agar plate. A10 µg meropenem disk is placed in the center of the test area. The test organism is streaked in a straight line from the edge of the disk to the edge of the plate.

Likewise *K. pneumoniae* positive and negative controls were similarly streaked. The plate was incubated overnight at 35±2°C in ambient air for 16–24 hours. After incubation, positive MHT was visible in a form of a clover leaf-like shape of *E. coli* growth along the test organism streak within the diffusion zone. Negative MHT was shown by no growth of Escherichia coli along the test organism streak within the diffusion zone (Basher, 2016).

2.2.6.4.2. Double disk synergy test (DDST)

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

2.2.6.4.3. EDTA-synergy test

EDTA inhibition of β-lactamase activity is used to differentiate a metallo β-lactamase from other β-lactamases.
To perform EDTA-disk synergy test an overnight culture of the test isolate is suspended in a McFarland broth (turbidity No. 0.5) and swabbed on a Mueller-Hinton agar plate. After drying, 10-µg imipenem disks together with a blank filter paper disk are placed on Mueller–Hinton agar plate 10 mm apart. 10 µL of 0.5 M EDTA solution was applied to the blank disk (i.e. approximately 1.5 mg/disk). After overnight incubation, the presence of an enlarged inhibition zone of imipenem indicates a positive EDTA-synergy test (Basher, 2016).

2.2.6.4.4. E tests

E tests with a ceftazidime or cefotaxime gradient at one end and a ceftazidime or cefotaxime plus clavulanate gradient at the other can be

used to detect ESBLs. They should be used in accordance with the manufacturer's package insert, which specifies a heavier inoculum than for the BSAC disc test method. ESBL production is inferred if the ratio of the MIC of cephalosporin alone to the MIC of cephalosporin plus clavulanate is eight or more.

E test containing a cefepime/clavulanate gradient are also available and the manufacturer suggests that these are suitable for detecting all ESBLs, though there is only limited supportive data, particularly for strains with very weak ESBLs, such as TEM-12 (David and Brown, 2005).

2.2.6.4.5. Combined disc methods

These depend on comparing the zones given by discs containing an extended-spectrum cephalosporin with clavulanate with those for identical discs without this inhibitor. If an ESBL is present, the zones are enlarged by the presence of the inhibitor. The United States Clinical Laboratory Standards Institute (CLSI; formerly NCCLS) recommends comparison of the zones given by cefotaxime 30 µg versus cefotaxime + clavulanate 30 + 10 µg and ceftazidime 30 µg versus ceftazidime + clavulanate 30 + 10 µg discs (David *et al*., 2005).

2.2.7. Treatment

The carbapenems (imipenem, meropenem, ertapenem, doripenem) are still the first choice of treatment for serious infections with ESBL-producing *E. coli and K. pneumoniae*. It has been reported that >98% of the ESBLproducing *E. coli, K. pneumoniae* and *P. mirabilis* are still susceptible to these drugs (Perez *et al*., 2007). But with the emergence of the carbapenem-resistant Enterobacteriaceae, the ‘‘magic bullet’’ is actually difficult to find.

There are some older drugs which can be used to treat the ESBL-producing *E. coli* or *K. pneumoniae* infections. Fosfomycin was reported of having admirable in vitro activity against the ESBL-producing *E. coli* or *K. pneumoniae*. In Hong Kong, most of the ESBL-producing *E. coli* isolates were reported to be sensitive to fosfomycin (Ho *et al*., 2010). Colistin is another choice which we can consider for the treatment of these organisms. Although once considered as quite a toxic antibiotic, it is a last resort that we

Can consider at the present moment as there is no new anti gram negative antibiotics available for the treatment of these multidrug resistant organisms. Other than ESBL-producing organisms, actually colistin is used in the treatment of multidrug resistant *P. aeruginosa*, carbapenem resistant *A. baumannii*. Close monitoring for the development of side effects can improve the safety margin when prescribing the drug. Tigecycline is also one of the drugs in the pipeline which can be considered for treatment (Perez *et al*., 2007).

2.2.8. Previous studies

In study conducted by Almugadam (2016) a total of 100 *E. coli* isolates were obtained, among which 43(43%) and 57(57%) were collected from male and female, respectively. Also the distribution of these samples among age group were children 14 (14%), adult 41 (41%) and geriatric 45(45%). All isolates were screened and confirmed for the presence of ESBL phenotypically with Double Disk Synergy Test, and tested for their susceptibility to non Beta-lactam antibiotics. Among the 100 *E. coli* isolates, only 35% were judged as ESBLs-positive strains. The frequency of ESBLs production was not significantly different among gender as their frequency were 30%(13/43) in male and 38%(22/57) in female; and

among age as their frequency were 29%(4/14) in children, 34%(14/41) in adult and 37%(17/45) in geriatric (Almugadam *et al*, 2016).

In study to detect extended spectrum β- lactamase (ESBL) in bacteria isolated from urine collected from urinary tract of infected pregnant women, a total of 100 midstream urine samples were collected from Omdurman Military Hospital from April to May 2013. Specimens were cultured onto cysteine- lactose- electrolyte-deficient (CLED) agar to identify urinary tract infection (UTI) causative bacteria by colonial morphology, Gram reaction and conventional biochemical tests. Out of 100 urine specimen; 56(56%) of samples gave significant bacterial growth (the Gram negative bacilli were 42(75%). The most frequent Gram-negative were *K. oxytoca* 15(26.8%) and *E. coli* 10(17.9%), while the most abundant Gram positive was *S. saprophyticus*. In The susceptibility test, 5 isolates were resistant to 3rdgeneration cephalosporins (ceftazidime, ceftriaone and cefotaxime) as follow: 2 *K. pneumoniae*, 2 *E. coli* and 1 *P. vulgaris*. ESBL production test done for all bacterial resistance to 3rd generation cephalosporin and then confirmed by combination test by using Double disc synergy test 5(11.9%) isolates gave positive result (Khalafalla and Hamed elnil, 2013).

A report from Qutar has shown that a total of 109 isolates (17.3 %) were confirmed as ESBL producers and all were sensitive to meropenem in routine susceptibility assays. Most of the ESBL producers (99.1 %) were resistant to amoxicillin/clavulanic acid and ceftriaxone and 93.6 % were resistant to cefepime (SidAhmed *et al*., 2016).

Study carried to determine prevalence and antimicrobial susceptibility pattern of extended spectrum β-lactamases producing *E. coli*, *K.*

pneumoniae and *P. mirabilis* in Khartoum Sudan. The Out of 162 clinical isolates, *E. coli*, *K. pneumoniae*, and *P. mirabilis* represented 44.4%, 38.9%, and 16.7% respectively. 39.5% were confirmed as ESBLs producers (Rasha and Hammad, 2016).

In study to evaluate emergence of ESBL among multi drug resistant *E. coli* and *Klebsiella* species causing nosocomial UTI. Hundred strains of multi drug resistant (MDR) *E. coli* and *Klebsiella* spp. causing nosocomial urinary tract infections (UTIs) from two main hospitals from Khartoum (Omdurman teaching hospital and Fedail Hospital) were included in this study. Susceptibility testing was performed against antibiotics commonly used in treatment of urinary tract infections. *E. coli*, *K. pneumoniae* and *K. oxytoca* (49%, 38% and 13% respectively) were among the studied isolates. β-Lactamase was produced by all isolates; high resistance level for 3rd generation cephalosporin was noticed. ESBLs were detected in high prevalence among all multi drug resistant *E. coli* and *Klebsiella* spp. isolates 53%. Reported a rate of 53% of ESBLs among *E. coli* and *Klebsiella* spp. isolates from Khartoum hospitals (Mekki *et al*., 2010).

Another study to investigate the resistance of *E. coli* and *K. pneumoniae* producing extended-spectrum β-lactamases (ESBL) and the genotyping of ESBLs among Sudanese patients with urinary tract infection .Seventy one and 109 isolates of *E. coil* and *K. pneumoniae* were collected from culturing of 542 mid-stream urine samples of UTI patients and screened for ESBL resistance using disc diffusion method. Results: Disc diffusion method showed that, 55 out of 71 *E. coli* and 95 out of 109 *K. pneumoniae* isolates were ESBL producing organisms. The resistance rates of *E. coli* and *K. pneumoniae* to 4 antibacterial agents were: Ceftazidime 77.5% and 68.8%, Cefotaxime 90.1% and 94.4%, Cefepime

77.5% and 69.7%, Aztreonam 91.5% and 87.2%, respectively (Ahmed *et al*., 2016).

In a study for PhD, Ali compared antibiotic susceptibilities of Enterobacteriaceae isolated in Khartoum Teaching Hospital and Soba University Hospital. He found that resistance of *E. coli* and *Klebsiella* spp. to ceftriaxone and ceftazidime in both hospitals ranged from 56.5% to 79% (Ali, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design

3.1.1. Type of study

This is a cross-sectional study.

3.1.2. Study area

This study was done at El obied city the capital of the North Kordafan State, Sheikan locality. Clinical specimens were collected from El obied Teaching Hospital. The practical part was carried out at the lab research of Sudan University of Science and Technology.

3.1.3. Study duration

The study was carried out in the period from March to September, 2018.

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 urinary tract infections and wound infections were included in this study.

3.2.2. Exclusion criteria

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

3.3. Sample size

A total of 100 patients were enrolled in this study.

3.4. Collection of specimens

The specimens were collected from patients by using standard microbiological procedures. Pus from wound was collected by sterile cotton swab and urine specimens were collected in wide mouthed container. All specimens were transported to microbiology laboratory of the hospital with minimum delay for culture and sensitivity tests.

3.5. Laboratory work

3.5.1. Culture

All specimens were cultured on MacConkey agar, CLED agar and Blood agar media. The sterility and the efficiency of the culture media were tested by incubating 5% of plates aerobically overnight at 37°C then checked for growth. Control strains were examined for growth on culture and sensitivity media. All reagents were pre-tested using control strains.

3.5.1. Purification of the isolates

The isolates were streaked on nutrient agar and incubated overnight at 37^{o}C. At the end of incubation period, discrete colony was picked up and checked for purity under microscope.

3.5.2. Identification of the isolates

3.5.2.1. Gram stain

Bacterial smear was prepared by transferring portion of discrete colony to a drop of normal saline. The smear was covered with crystal violet stain for 30-60 second, rapidly washed the stain with clear water, then the

smear was covered with lougou's iodine for 30-60 seconds, washed of iodine with clear water, decolorized rapidly (few seconds) with acetone-alcohol, washed immediately with clean water, then the smear was covered with sufranine for minutes, washed off the stain with clear water, wiped back of the slid clean and placed in draining rack for the smear to air dry. The smear was examined microscopically with oil immersion objective to report bacterial Gram reaction and cell shape. Gram-positive bacteria; stained dark purple, Gram-negative bacteria; stained red (Cheesbrough, 2000).

3.5.2.2. Biochemical tests

a) Catalase test

The differentiation between staphylococci (which produce catalase) and streptococci (non catalase production) was made by catalase test. Catalase acts as catalyst in the breakdown of hydrogen peroxide to oxygen and water **(**Cheesbrough, 2006).

Using sterile wooden stick, suspected colony was immersed in tube containing 2ml of 3% hydrogen peroxide. A positive result was indicated by production of air bubbles. A negative indicated by no change on tube.

b) Mannitol fermentation test

Mannitol salt agar was used for identifying staphylococci species, which are able to grow on agar containing 70-100g/L sodium chloride. Some species of staphylococci are able to ferment mannitol and other cannot ferment.

The test done by inoculating organism under test on mannitol salt agar medium which contain phenol red as indicator, and then the plate was

incubated at 37ºC for 24 hours. Fermenting mannitol: medium turns yellow (Cheesbrough, 2000).

c) Indole test

The tested organism was inoculated into peptone water and incubated at 37ºC for overnight; the kovac's reagent was added. If there is pink ring the result was indicated positive. If there is no pink ring in surface the result indicated negative (Cheesbrough, 2006).

d) Citrate utilization test

In this test the organism has ability to use citrate as only source of carbon. By straight wire a part of tested colony were emulsified in Kosser citrate medium and incubated for 24 hours in 37ºC. Positive: blue color. Negative: no change (Cheesbrough, 2006).

e) Urease test

In the test organism produce urease enzyme break down urea and produce ammonia, which make pH media alkaline, in the presence of phenol red as indicter. The tested organism was inoculated in the christensen's urea agar and incubated for 24 hours in 37ºC. Positive: pink color. Negative: no change (Cheesbrough, 2006).

f) Fermentation of sugar and production of gas and H_2S

The organism under test was inoculated by sterile straight wire by stapping on the butt, then blocked the pore and streaked slop part and inoculated for 24 hours in 37ºC. Glucose fermentation was detected by yellow butt, lactose fermentation was detected by yellow slop, while gas production was detected in the end of the tube and H_2S production blacking in the media (Cheesbrough, 2006).

3.5.2.3. Antimicrobial susceptibility test

This was done by Kirby-Bauer disk diffusion technique according to Clinical Laboratory Standards Institute (CLSI) guidelines. The antibiotics which were tested included Ceftazideime, Amoxicillin, Gentamicin, Ciprofloxacin, Nitrofurantoin, Imipenem, Penicillin, Amikacin (Muhammad and Swedan, 2015).

Culture medium

Sterilized Mueller-Hinton agar was prepared. Cooled to 45-50 ^{o}C, and poured in sterile dry Petri dish on level surface, to depth of 4mm. The presence of any excess surface moister on the medium was removed by keeping the plates inverted in an incubator at 35-37 ^{o}C.

Preparation of inoculums

The inoculums were prepared by direct colony suspension method. Five well selected colonies (similar appearance) of the organism to be tested were touched with sterile loop and transferred to sterile saline. The inoculum turbidity was adjusted to McFarland standard turbidity (Cheesbrough, 2002).

Seeding of the plates

Cotton wool swab previously immersed in suspension of the test organisms was rotated and squeezed to remove the excess and streaked evenly on dry Muller- Hinton agar (Oxoid Co. Ltd., U.K.). Seeded plates were left for few minutes at room temperature with lid closed (Vandopitte *et al*., 199l).

Antimicrobial disks application

By using a pair of sterile forceps, antibiotic disk were applied and evenly distributed on the inoculated plate. The plate was inverted and incubated aerobically at 37 ºC for 18-24 hours according to CLSI guidelines. *E. coli* ATCC 25922 was used as control strain.

Reading and interpretation

The diameter of each zone of inhibition (including the diameter of the disk) was measured to nearest millimeter using ruler. The susceptibility of isolates was reported according to manufacture standard zone size interpretative manual. Sensitive organisms were when the zone of inhibition was equal or greater than the standard.

3.5.2.4. Detection of β-Lactamase

All isolates found resistant to β-lactam antibiotics were further tested for detection of ESBLs using the Double Disk Synergy Test (DDST) and Combined Disk Method as recommended by the CLSI guidelines.

Double Disk Synergy Test (DDST)

Standardized inoculum of bacterial suspension equivalent to 0.5 McFarland standard turbidity of each isolate was inoculated on Mueller-Hinton agar plates using a sterile cotton swab. Then with sterile forceps the disk of amoxicillin-clavulanic acid (30ug) was placed at centre of plate and the disks of cefotaxime (30ug) and ceftazidime (30µg) were placed (centre to centre) at distance 20 mm from amoxicillin-clavulanic acid. After incubation at 37ºC for 18 hours aerobically, a clear extension of the edge of the inhibition zone of cefotaxime and ceftazidime towards amoxicillin-clavulanic acid disk was interpreted as positive for ESBL production (Mekki1 *et al*., 2010).

Combined disc methods

Standardized inoculum of bacterial suspension equivalent to 0.5 McFarland standard turbidity of each isolate was inoculated on Mueller-Hinton agar plates by using a sterile cotton swab. Then with sterile forceps the disk of ceftazidime and ceftazidime + clavulanate were placed. After incubation at 37ºC for 18 hours aerobically. If an ESBL is present, the zones are enlarged by the presence of the inhibitor (David and Brown, 2005).

3.6. Data analysis

Data were computed and analyzed by using statistical package for social science (SPSS) computer software version 16.0, to check the statistical significance the p-value considered significant was <0.05.

GHAPTER FOUR

RESULTS

4. Results

A total of 100 clinical specimens were collected from both males and females attended El-obied Teaching Hospital. The age of participant range from 4-65 years.

The gender incidence of patients investigated were 32(32%) males, and 68(68%) were females, as shown in Table (1). The largest number of specimens (72/72%) was collected from patients aged 13-45 years (Adult), while the fewest number of specimens (10/10%) was collected from patients aged 4-12 years (Children) as shown in Table (2).

The specimens were cultured on MacConkey agar, CLED agar, and Blood agar media. The number of specimens that gave growth was 60 specimens (60%); while 40 specimens (40%) showed no bacterial growth. The isolates were identified by colonial morphology, Gram stain and biochemical tests. The isolates from urine specimens were 40 organisms (66.7%), and 20 organisms (33.3%) were isolated from wound swabs. *K. pneumoniae* was the commonest pathogen (15.7%) among urine isolates; while *Proteus* species was the predominant pathogen (37.5%) among wound swabs isolates. The distribution of isolated organisms was 54/60 (90%) Gram-negative bacilli and 6/60 (10%) Gram-positive cocci (Table 3).

All isolated bacteria were subjected to antimicrobial sensitivity test. The results revealed that the highest resistance rates were observed to Amoxicillin (85%) followed by Ceftazideime (64%), and Nitrofurantoin (50%). While the highest antimicrobial activities against bacterial isolates

were observed to Impenem (100%) followed by Gentamicin (98.4%), Ciprofloxacin (91.7%) and Amikacin (91.7%), as shown in Table (4).

Twenty eight (n=28) bacterial isolates resistant to third generation cephalosporin (Ceftazideime) were further tested using the Double Disk Synergy Test (DDST) and Combined Disk Method to detect the ESBLs. Only ten (35.7%) were found to be ESBLs producer by Double Disk Synergy Test (DDST) and when tested by the Combined Disc Methods the ESBLs producer were 15(53.6%) as shown in Table (6). The majority of ESBL producers were from urine (n=13/ 86.6%) followed by wound swabs (n=2/13.3%) and *E. coli* was the most ESBLs producer

The frequency of organisms isolated from males patients compared with that isolated from females patients was found insignificant ($p = 0.07$), as shown in Table (7), while the frequency of ESBLs producer in different age groups was found significant ($p = < 0.03$), as shown in Table (8).

The most ESBLs producer was collected from patients who were housekeeper (40/ 66.6%) and have basic level education (40/66%) as shown in Table (9) and Table (10).

Table 1. Distribution of clinical specimens according to the gender

Gender	Specimens		Total
	Urine	Wound swabs	
Male	18	14	32
Femal	58	10	68
Total	76	24	100

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

Age groups	specimens		Total
	Urine	Wound swabs	
(up to12 years)	7	3	10
(13 to 45 years)	60	12	72
(more than 45 years)	9	9	18
Total	76	24	100

Table 3: Organisms isolated according to type of specimens

Organism isolated	Specimens		Total
	Urine	Wound swabs	
E. coli	10(13.1%)	5(20.8%)	15
K.pneumoniae	12(15.7%)	5(20.8%)	17
Proteus spp.	9(11.8%)	9(37.5%)	18
P. aeruginosa	3(3.9%)	1(4.1%)	4
S. aureus	6(7.8%)	0(0%)	6
Total	40	20	60

Table 4: Profile of antibiotics sensitivity and resistance against pathogenic Bacteria

Antibiotic	Bacteria	Sensitive %	Resistance%
Gentamicin	*E.c.*(n=15)	100	0
	Ps. a.(n=4)	100	0
	K.pn.(n=17)	100	0
	Pr. spp.(n=18)	94.4	5.6
	S.a.(n=6)	100	0
Ciprofloxacin	*E.c.*(n=15)	66.6	33.4
	Ps. a.(n=4)	100	0
	K.pn.(n=17)	94.1	5.9
	Pr. spp.(n=18)	88.8	11.2
	S.a.(n=6)	100	0
Ceftazideime	*E.c.*(n=15)	33.3	66.7
	Ps. a.(n=4)	50	50
	K.pn.(n=17)	64.8	35.2
	Pr. spp.(n=18)	44.4	55.6
	S.a.(n=6)	14.2	85.8
Impenem	*E.c.*(n=15)	100	0
	Ps. a.(n=4)	100	0
	K.pn.(n=17)	100	0

	Pr. spp.(n=18)	100	0
	S.a.(n=6)	100	0
Amikacin	*E.c*.(n=15)	100	0
	Ps. a.(n=4)	100	0
	K.pn.(n=17)	88.2	11.8
	Pr. spp.(n=18)	88.8	11.1
	S.a.(n=6)	83.3	16.6
Nitrofurantoin	*E.c*.(n=15)	66.6	33.4
	Ps. a.(n=4)	50	50
	K.pn.(n=17)	52.9	47.1
	Pr. spp.(n=18)	50	50
Penicillin	*S.a*.(n=6)	0	100
Amoxicillin	*E.c*.(n=15)	0	100
	Ps. a.(n=4)	0	100
	K.pn.(n=17)	5.5	94.5
	Pr. spp.(n=18)	11.7	88.2
	S.a.(n=6)	16.6	83.3

Key: *E.c. = Escherichia coli; K.pn. = Klebsiella pneumoniae;* *Pr*.spp= *Proteus* species*; Ps.a=Pseudomonas aeruginosa;* S.a =*Staphylococcus aureus*

Table 5: Cephalosporine sensitivity pattern

Organisms isolated	**Sensitivity and resistant to cephalosporin**		**Total**
	Sensitive	**Resistant**	
E. coli	5(33.3%)	10(66.7%)	15
K. pneumoniae	11(64.7%)	6(35.3%)	17
Proteus spp	8(44.4%)	10(55.5%)	18
P. aeruginosa	2(50%)	2(50%)	4

Total	32	28	60

Table 6: Percentage of ESBLS producer and non-ESBLS producer among bacteria

Organisms isolated	ESBLs				Total
	DDST		CDM		
	positive	Negative	Positive	Negative	
E. coli	5(33.3%)	10(66.7%)	7(46.6%)	8(53.4%)	15
K. pneumoniae	3(17.6%)	14(82.4%)	3(17.6%)	14(88.2%)	17
Proteus spp	2(11.1%)	16(88.8%)	4(22.2%)	14(77.8%)	18
P. aeruginosa	0(0%)	4(100%)	1(25%)	3(75%)	4
Total	10	50	15	45	60

(DDST): Double Disk Synergy Test; (CDM): Combined Disk Methods.

Table 7: Distribution of positive result according to the gender

Gender	ESBLS				Total
	DDST		CDM		
	Positive	Negative	positive	Negative	
Male	3(12%)	22(88%)	4(16%)	20(80%)	25
Female	7(20%)	28(80%)	11(31.4%)	25(71.4%)	35
Total	10	50	15	45	60

(P.value=0.07)

Table 8: Distribution of positive result according to the age groups

Age group	ESBLS				Total
	DDST		CDM		
	positive	Negative	positive	Negative	
Children(up to12 years)	1(12.5%)	7(87.5%)	3(37.5%)	5(62.5%)	8
Adult(13-45 years)	8(21.6%)	29(78.4%)	11(29.7%)	26(70.3)	37
Elderly(more than 45years)	1(6.7%)	14(93.3%)	1(6.7%)	14(93.3%)	15
Total	10	50	15	45	60

(P.value=0.03)

Table 9: Distribution of positive result according to the education level

Level of education	ESBLS				Total
	DDST		CDM		
	positive	Negative	positive	Negative	
Basic school	8(20.5%)	32(80%)	13(32.5%)	27(67.5%)	40
Secondary school	2(12.5%)	14(87.5%)	2(12.5%)	14(87.5%)	16
University	0(0%)	4(100%)	0(0%)	4(100%)	4
Total	10	50	15	45	60

(P.value=0.05)

Table 10: Distribution of positive result according to the occupation

Occupation	ESBLS				Total
	DDST		CDM		
	Positive	Negative	Positive	Negative	
Farmer	1(16.7%)	5(83.3%)	1(16.7%)	5(83.3%)	6
Housekeeper	7(17.5%)	33(82.5%)	11(27.5%)	29(72.5%)	40
Student	2(25%)	6(75%)	3(37.5%)	5(62.5)	8
Employer	0(0%)	6(100%)	0(0%)	6(100%)	6
Total	10	50	15	45	60

(P.value=0.06)

CHAPTER FIVE

DISCUSSION

5.1. Discussion

Antimicrobial resistance was recognized as an increasingly global problem, especially among Gram-negative bacteria (Slama, 2008). Since there are no implemented polices in Sudan to control antibiotics usage and antibiotics overuse self medication has resulted in a wide scale antimicrobial resistance in our hospitals (Elhag, 2013).

Based on the results of this study, the distribution of ESBL among *E. coli*, *K. pneumoniae*, *Proteus* spp. and *P. aeruginosa* was 33.3%, 17.6%, 11.1% and 0% respectively. This finding is lower than those obtained from the study done by Rasha (2016) in Khartoum, Sudan, in which *E. coli* , *K. pneumoniae* and *Proteus* spp. were 44.4%, 38.9%, and 16.6% respectively and also lower than study done by Basher (2016) who reported *K. pneumoniae, E. coli* and *Proteus* spp. were 8.7%, 38.1%, and 29.6% respectively.

In this study urine was the main source of ESBLs producing isolates (86.6%). This finding is in-line with Akbar (2007) who reported that (70%) of urine was the main source of ESBLs.

The most ESBLs producer was *E. coli*. Similar result was obtained by Omer (2013) in Sudan.

In this study the ESBLs producers isolated from males (12%) and (20%) from females. These findings are lower than that obtained by Almugadam (2016) who reported ESBLs producers were 30% in males and 38% in females and lower than that reported by Vidhya (2013) who reported ESBLs 47.22% in males, and 52.77% in females.

The frequency of ESBLs among age groups were 12.5% in Children, 21.6% in Adult, and 6.7% in Elderly. These findings are lower than results obtained by Almugadam (2016) who reported ESBLs producer among Children 29%, 34% in Adult, and 37% in Elderly.

The results of the present study are in agreement with other study done by Almugadam (2016) who reported that the ESBLs producers were sensitive to impenem (100%).

5.2. CONCLUSION

The study concluded that there is high ratio of ESBLs among isolated bacteria in El-obied city, Sudan. The most ESBLs producer is *E. coli.* All ESBLs producers are sensitive to impenem.

5.3. RECOMMENDATIONS

1. A larger sample size should be tested to cover a wider range of isolates.
2. Other specific tests for detection of EBSL enzymes should be used, such as PCR, and different types of PCR assays should be coupled with each other to increase the sensitivity for enzymes detection.
3. Detection of EBSL producers should be introduced as routine tests in microbiology labs for rapid detection of resistant isolates and to control their spread, especially for newly admitted patients to the hospitals.
4. Prevention and control programs of cephalosporine resistant Gram-negative bacteria should be performed to prevent the spread of EBSL producers, which includes appropriate use of antimicrobials and facility-level prevention strategies, as recommended by the CDC.

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5.6. Appendices

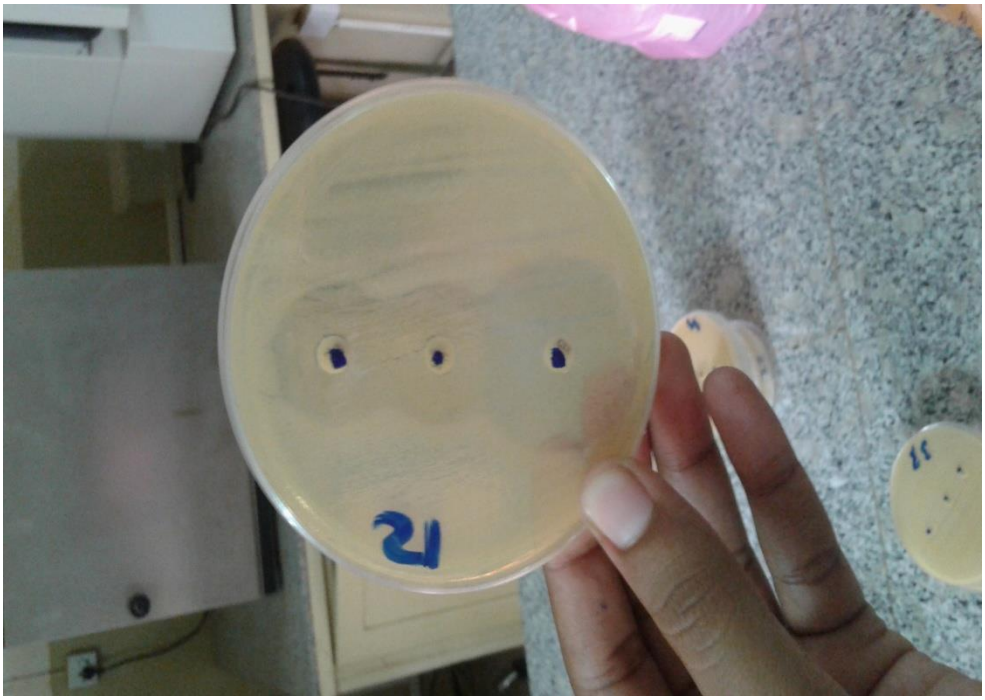

Figure1. Positive result of Double Disk Synergy Test

Figure2. Negative result of Double Disk Synergy Test

Figure 3. Positive result of Combined Disk Methods

Figure 4. Negative result of Combined Disk Methods