CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Since the introduction of broad spectrum cephalosporins in clinical use, leading to increase in development and emergence of resistance by the misuse and overuse of these agents (Hassan *et al.,* 2013).

β- lactamases are a members of group that contains hundreds enzymes which inactivate β- lactam ring of various members of β- lactam group of antimicrobial agents (Kanneth and George , 2004).

The emergence of multi-drug resistance Gram-negative bacteria is a major concern in hospital settings in many parts of the world. Infections caused by these pathogens have become significantly challenging over the past two decades, particular in the developing countries and are associated with high morbidity and mortality rates as well as protracted hospital stay (Singh and Manchanda, 2017).

Detection of extended spectrum β-lactamases (ESBLs) is a challenge for microbiology laboratories because routine methods for monitoring a decrease in susceptibility to oxyimino-cephalosporins are not sensitive enough to detect all ESBL producing strains (Ho *et al.,* 1998).

1.2. Rationale

The antibiotic resistance is a world-wide health problem; it leads to more of failure in treatment for bacterial infections. β-lactam antibiotics are most common drugs used for several infections. In the last years many studies showed that there is an increasing rate of failure of treatment in the different states of Sudan. The objective of this study aimed to detect the β-lactamases production in different isolates in El Hawata, Al Gadaref State to determine efficacy of used antibiotics in treating different bacterial infections.

1.3 Objectives

1.3.1 General objective

To detect extended spectrum beta-lactamases in different clinical isolates.

1.3.2 Specific objectives

1. To collect different clinical specimens from patients hospitalized in El Hawata Rural Hospital.
2. To isolate and identify bacteria obtained from different clinical specimens.
3. To perform antimicrobial susceptibility tests.
4. To detect the presence of ESBLs using double disc confirmatory technique.

CHAPTER TWO

LITERATURE REVIEW

2.1. Antibiotics

An antibiotic (Greek: anti, against + biosis, life) is a substance that produced by fungi or bacteria, that inhibit the growth of other microorganisms (Karen *et al.*, 2011). It may be natural, semi synthetic or synthetic compounds use for treatment of bacterial infections; they act either by killing or inhibit the growth of bacteria. The antibiotics have been divided into different families according to their chemical structure, mode of action and the site of action (Fisher *et al.,* 2005).

2.2. Classification of antibiotics

Antibiotics are classified according to their target on bacteria into; antibiotics affecting cell wall synthesis (this group contain a β-lactam antibiotics), antibiotics affecting cell membrane, antibiotics affecting cell membrane, antibiotics affecting protein synthesis, antibiotics affecting folic acid synthesis and last group affecting nucleic acid (DNA or RNA) synthesis (Cowan *et al.,* 2016).

2.2.1. Beta-lactam antibiotics

β-lactam antibiotics are a class of broad spectrum antibiotics, consisting of all antibiotics that contain a beta-lactam ring in their molecular structures, this include penicillin derivatives (penams), cephalosporin'

(cephems), monobactams and carbapenems (Holten and Onusko, 2000). Beta-lactam antibiotics are bactericidal and act by inhibiting the synthesis of peptidoglycan layer of bacterial cell walls, the peptidoglycan is important for the cell wall integrity, especially in Gram-positive organisms (Fisher *et al.,* 2005).

2.3. Antibiotic resistance

Since the mid-1900s, an increasing number of bacterial species have become resistant to antibiotics. Resistance means that the bacterial cells are not effectively inhibited or killed with the use of antibiotics allowing the resistant cells to continue multiplying in the presence of therapeutic levels of an antibiotic (Jeffrey, 2011).

Bacterial resistance to antibiotics can develop through four major mechanisms as summarized below; which include altered metabolic pathway, antibiotic modification, reduced permeability active export of antibiotics or target modification (Jeffrey, 2011).

2.3.1. Resistance to β-lactam antibiotics

The bacteria can develop a resistance against the β-lactam group of antibiotics by production of the enzyme β-lactamase (antibiotic modification); the enzyme hydrolysis the β -lactam ring of the antibiotic rendering the antibiotic ineffective (Leonardo *et al.,* 2010)

Summary of bacterial resistance mechanisms

Antibiotic Target	Antibiotic Class	Resistance Mechanisms
Inhibition of cell wall synthesis	Penicillins, cephalosporins, carbapenems, vancomycin	Altered wall composition; drug destruction by β -lactamases
Membrane permeability and transport	Polypeptide antibiotics	Altered membrane structure
Inhibition of DNA synthesis	Fluoroquinolones	Inactivation of drug; altered drug target
Inhibition of RNA synthesis	Rifampin	Altered drug target
Inhibition of protein synthesis	Aminoglycosides, chloramphenicol, tetracyclines, macrolides, clindamycin, streptoGramins, oxazolidinones	Altered membrane permeability; drug pumping; antibiotic inactivation; altered drug target
Inhibition of folic acid synthesis	Sulfonamides	Alternate metabolic pathway; altered drug target

β-lactamases destroy the molecular antibacterial properties there by breaking and opening the common element in their molecular structure β-lactam (Bush and Mobashery, 1995).

The production of β -lactamases by a bacterium does not necessarily rule out all treatment options with β-lactam antibiotics; in some instances β-lactam antibiotics may be co-administrated with β-lactamase inhibitor, for

example, Augmentin is made of amoxicillin (β-lactam antibiotic) and clavulanic acid (β-lactamase inhibitor) the clavulanic acid is designed to overwhelm all β-lactamase enzymes, and effectively serve as antagonist so that the amoxicillin is not affected by the β-lactamase enzymes. Other β-lactamase inhibitors such as boronic acid are being studied in which they are irreversibly bound to the active site of β-lactamases, this is a benefit over clavulanic acid and similar β-lactam competitors, because they cannot be hydrolyzed, and there for rendered useless (Leonard *et al.,* 2013).

2.3.2. β -lactamases

β-lactamases are a group of enzymes that produced by Gram positive as well as Gram negative bacteria; their production is either to be chromosomally encoded or plasmid mediated (Cowan *et al.,* 2016).

Classification schemes have been created based on molecular weight (into class A, B, C and D), substrate profile and inducibility (either to be inducible or constitutively) (Kanneth and George, 2004).

2.3.2.1. Penicillinase is a specific type of β-lactamases showing specificity for penicillins, acting by hydrolyzing the β -lactam ring. Molecular weights of the various penicillinases tend to cluster near 50kilodaltons. Penicillinase was the first β-lactamase to be identified; it was first isolated by Abraham and Chain in 1940 from Gram negative *E.*

coli even before penicillin entered clinical use (Abraham and chain 1940).

2.3.2.2. TEM β-lactamases (class A) TEM-1 is the most commonly encountered β-lactamases in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Paterson *et al.,* 2005). The term TEM comes from the name of the Athenian patient (Temoniera) from which the isolate was recovered in 1963 (Yousif *et al.*, 2018).

2.3.2.3. SHV β-lactamases (class A) shares 68 percent of its amino acids with TEM-1 and has a similar overall structure; The SHV-1 β-lactamases is most commonly found in *K. pneuomniae* and is responsible for up to 20% of the plasmid mediated ampicillin resistance in this species. More than 60 SHV varieties are known. SHV-5 and SHV-12 are among the most common (Paterson and Bonomo, 2005).

2.3.2.4. CTX-M β-lactamases (class A) these enzymes were named for their greater activity against cefotaxime than other oxyimino-β-lactam substrate. These enzymes are not very closely related to TEM and SHV β-lactamases in that they show only 40% identity with these two commonly isolated β-lactamases (Fisher *et al.,* 2005).

2.3.2.5. Extended-Spectrum β-lactamases (ESBLs)

In the mid of 1980's, a new group of β-lactamase enzymes to the extended spectrum β-lactams were detected (Bush and Mobashery,

1995).According to the Bush and Jacoby scheme (Bush and Jacoby, 2010) ESBLs enzymes can be divided into four main groups: group1 cephalosporinases which are not inhibited by clavulanic acid, group 2 are a broad spectrum enzymes which comprise the largest group and are generally inhibited by clavulanic acid except the 2d and 2f subgroups (Dhillon and Clark, 2012); subgroups of enzymes namely 2a, 2b, 2be, 2br, 2c, 2d, 2e and 2f, were defined based on the rates of hydrolysis of carbenicillin, cloxacillin, extended-spectrum β-lactams ceftazidime, cefotaxime, or aztreonam, and of inhibition profile by clavulanic acid respectively, enzymes that are inhibited by the metal-chelating agent EDTA are classified as group 3, finally Group 4 consists of other β-lactamases that are not inhibited by clavulanic acid (Ambler *et al.*, 1991; Ambler, 1980).

However, most ESBLs are allocated to group 2be which can hydrolyse penicillins, monobactams, and cephalosporins and are inhibited by clavulanic acid; they are class A according to the Ambler molecular scheme. CTX-M genotype still suits the former criteria for group1be enzymes (Dhillon and Clark, 2012).

2.3.3. Classification of ESBLs

These enzymes can be classified according to two general schemes; the Ambler molecular classification scheme and the Bush-Jacoby-Medeiros functional classification system (Bush *et al.*, 1995).

Classification scheme for β-lactamases proposed by Ambler has found common usage (Ambler 1980). Ambler classifies these enzymes into four classes, A, B, C, and D (Ambler, 1980). Classes A, C, and D have evolved dependence on an active site serine as their key mechanistic feature; class B enzymes are zinc dependent and hence different. The catalytic process for turnover of the members of the former group involves acylation at the active site serine by the β-lactam antibiotic, followed by deacylation of the acyl-enzyme species. It is noteworthy that these enzymes do not share any sequence homologies, structural similarities, or mechanistic features with serine or zinc-dependent proteases (Beceiro and Bou, 2004; Bush and Mobashery, 1998).

Class A β-lactamase generally prefers penicillins as substrates, whereas class C enzymes turn over cephalosporins better, class B enzymes can hydrolyze a broad range of substrates including carbapenems, which resist hydrolysis by most of the other classes of enzymes, class β-lactamases, on the other hand hydrolyze oxacillin-type β-lactams efficiently. Classes A and C of β-lactamases are the most common and the second most common enzymes, respectively (Beceiro and Bou, 2004; Bush and Mobashery, 1998).

2.3.3.1. Class A β-lactamases

Generally, class A β-lactamase enzymes are susceptible to β-lactamase inhibitors such as clavulanic acid, tazobactam and to a lesser extent

sulbactam; However the *K. pneumoniae* carbapenemase KPC may be an exception to this generalization (Papp-Wallace *et al.*, 2009). The first plasmid mediated member of this class was described in *E. coli* in 1963, and was named TEM (Datta and Kontomichalou, 1965). SHV which is another common β-lactamase detected primarily in *K. pneumonia*e (Matthew *et al.*, 1979). TEM and SHV are common β-lactamases described in clinical isolates of *E. coli* and *K. pneumoniae*, primarily responsible for urinary tract, bloodstream, and hospital-acquired respiratory tract infections (Buynak, 2006).

2.3.3.2. Class B β-lactamases

Class B enzymes can hydrolyze a broad range of substrates including carbapenems, which resist hydrolysis by most of the other classes of enzymes; class β-lactamases on the other hand hydrolyze oxacillin type β-lactams efficiently (Beceiro and Bou, 2004).

2.3.3.3. Class C β-lactamases (AmpC)

AmpC type β-lactamases are commonly isolated from extended spectrum cephalosporins resistant Gram negative bacteria. AmpC β-lactamases (also termed class C or group 1) are typically encoded on the chromosome of many Gram negative bacteria including *Citrobacter, Serratia* and *Enterobacter* species where its expression is usually inducible; it may also occur on *Escherichia coli* but is not usually

inducible, although it can be hyper expressed. AmpC type β-lactamases may also be carried on plasmids (Taslima, 2013).

2.3.3.4. Class D β-lactamases (OXA)

OXA β-lactamases were long recognized as a less common but also plasmid mediated variety that could hydrolyze oxacillin and related antistaphylococcal penicillins. These β-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The OXA type β-lactamases confer resistance to ampicillin and cephalothin also they characterized by their high hydrolytic activity against oxacillin and cloxacillin and they are poorly inhibited by clavulanic acid. Amino acid substitutions in OXA enzymes can also give the ESBL phenotype (Taslima, 2013).

2.3.3.5. Others

Other plasmid mediated ESBLs such as PER, VEB, GES, and IBC β-lactamases has been described but are uncommon and have been found mainly in *Ps. aeruginosa* and at a limited number of geographic sites. PER-1 in isolates in Turkey, France, and Italy; VEB-1 and VEB-2 in strains from Southeast Asia; and GES-1, GES-2, and IBC-2 in isolates from South Africa, France, and Greece (Taslima, 2013).

2.4. Epidemiology

The epidemiology of ESBLs is quite complicated; there are certain levels to consider, which includes the wider geographical area, the country, the

hospital, the community and the host (in most cases a single patient or a healthy carrier). Moreover, these are bacteria (*E. coli* is more endemic, and *K. pneumoniae* is more epidemic) and their mobile genetic elements (usually plasmids). Additionally there are various reservoirs including the environment (e.g. soil and water), wild animals, farm animals, and pets. The final component includes transmission from food and water; also via direct or indirect contact (person to person) (Carattoli, 2008).

The first ESBL to be identified was found in Germany in 1983, but it was in France in 1985 and in the United States at the end of the 1980s and the beginning of the 1990s that the initial nosocomial outbreaks occurred (Rice *et al*., 1990).

New TEM and the SHV enzymes are still emerging in Europe, and distinct epidemic clones have been found, for example *Salmonella* isolates with TEM-52 in Spain (Fernandez *et al.,* 2006) and *Escherichia coli* isolates with SHV-12 in Italy (Perilli *et al.*, 2011). Isolates with the CTX-M-9 group are common in Spain and strains with the CTX-M-3 enzymes have been described chiefly in Eastern Europe, although clones producing the CTX-M group-1 (including the CTX-M-15 type) are the most widespread throughout Europe (Coque *et al*., 2008; Canton and Novdmam, 2008).

A study conducted at the National Public health laboratory (NPHL), Kathmandu, Nepal reported that 31.57% of *E. coli* were confirmed as

ESBLs producers, these isolates further exhibited co resistance to several antibiotics (Thakur *et al.,* 2013).

Data on ESBL producing Enterobacteriaceae in the countries of the Middle East are extremely worrisome and this region might indeed be one of the major epicenters of the global ESBL pandemic (Mohamed AlAgamy *et al.,* 2006).

Investigation conducted in Saudi Arabia in 2004–2005 showed that 10% of clinical urinary *E. coli* isolates from inpatients and 4% of such isolates from outpatients were ESBL producers (Khanfar *et al.,* 2009).

Moubareck and colleagues analyzed fecal samples in Lebanon in 2003 and noted that ESBL carriage differed somewhat between patients (16%), healthcare workers (3%), and healthy subjects (2%), also there was a predominance of the CTX-M-15 enzyme (83%) (Moubareck *et al*., 2005). Other researchers in Lebanon (Khanfar *et al.,* 2009) observed that the proportion of ESBL producing isolates was significantly larger among inpatients (15.4%) than in outpatients (4.5%). Moreover, data collected over three years in Kuwait showed that the levels of ESBLs were lower in community isolates of *E. coli* (12%) than in the corresponding hospital isolates (26%) (Al Benwan *et al.,* 2010).

In other recent studies authors observed ESBL rates of 46% and 50% in out and inpatients respectively (Sankar *et al*., 2012).

Also in 2016 Tabar *et al.,* in Semnan, Iran collected 2618 urine samples; one hundred nine samples were identified as *E. coli* (4.16%), twenty one 26.6% of *E. coli* were ESBL positive and 73.4% were ESBL negative. Twenty six (89.65%) of the positive samples were females and three (10.34%) were males and the average of age was 32 years old. There was 100% susceptibility to imipenem, twenty (68.97%) out of 29 isolates were positive for the CTX-M gene as detected by PCR.

Mekki *et al*. isolated hundred strains of multi drug resistant *Escherichia coli* and *Klebsiella* species causing nosocomial UTI from two main hospitals in Khartoum 2010 (Omdurman teaching hospital and Fedail hospital). Susceptibility testing was performed against antibiotics commonly used in treatment of UTI. *E. coli* was among the study isolates; β-lactamase was produced by most of isolates, high resistance level for third generation cephalosporin was noticed. ESBLs were detected in high frequency among all multi drug resistant *E. coli* and *Klebsiella* species isolates (53%).

2.5. Method for detection of ESBLs

Many clinical microbiology laboratories make no effort to detect ESBL production by Gram negative bacilli, or are ineffective at doing so. In a 1998 survey of 369 American clinical microbiology laboratories, only 32% (117 of 369) reported performing tests to detect ESBL production by *Enterobacteriaceae*. A subsequent survey of laboratory personnel at 193

hospitals actively participating in the National Nosocomial Infections Surveillance system showed that only 98 (51%) correctly reported a test organism as an ESBL producer. In a study from Europe, just 36% of 91 ESBL producing *Klebsiella* spp were reported by their original clinical laboratories as cefotaxime resistant (Paterson and Bonomo, 2005).

The most liberal interpretation of cephalosporin susceptibility has been that of the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards). The CLSI break-points for susceptibility of members of the *Enterobacteriaceae* to extended-spectrum cephalosporins and aztreonam were developed in the early 1980s (Paterson and Bonomo, 2005).

CLSI recommended methods for ESBLs detection includes screening and confirmatory tests as following:

2.5.1. Screening methods for ESBL detection

There are two main methods: i) Disk diffusion methods; the CLSI has proposed disk diffusion methods for screening for ESBL production by klebsiellae, *Escherichia coli*, and *Proteus mirabilis*. Laboratories using disk diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters which indicate a high level of suspicion for ESBL production. Cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone is used. However, the use of more than one of these agents for screening improves the sensitivity of

detection. If any of the zone diameters indicate suspicion for ESBL production, phenotypic confirmatory tests should be used to ascertain the diagnosis. The second is (ii) Screening by dilution antimicrobial susceptibility tests; The CLSI has proposed dilution methods for screening for ESBL production by klebsiellae and *Escherichia coli*. Ceftazidime, aztreonam, cefotaxime, or ceftriaxone can be used at a screening concentration of 1 µg/ml. Growth at this screening antibiotic concentration is suspicious of ESBL production and is an indication for the organism to be tested by a phenotypic confirmatory test (Paterson and Bonomo, 2005).

2.5.2. Phenotypic Confirmatory Tests for ESBLs Production

i) Cephalosporin/clavulanic acid combination disks; the CLSI advocates use of cefotaxime (30 µg) or ceftazidime disks (30µg) with or without clavulanic acid (10 µg) for phenotypic confirmation of the presence of ESBLs in Klebsiella spp and *E. coli*. Prior to the combination disks becoming available, it was recommended that clavulanic acid solution be applied to the cephalosporin disks within one hour before they are applied to the agar plates. The CLSI recommends that the disk tests be performed with confluent growth on Mueller-Hinton agar. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanic acid disk is taken to be phenotypic confirmation of ESBL production (Paterson and Bonomo, 2005).

In an evaluation of 139 *Klebsiella pneumoniae* isolates that met the National Committee for Clinical Laboratory Standards screening criteria for potential ESBL production, Steward and his colleagues at 2004were found that 84% (117 of 139) met the criteria for a positive phenotypic confirmatory test. Of the 117 isolates, 104 (89%) met the criteria for a positive phenotypic confirmatory test with both ceftazidime and cefotaxime, 11 (9%) with ceftazidime only and 2 (2%) with cefotaxime only (Paterson and Bonomo, 2005).

ii) Broth microdilution; phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25 to 128 μg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4 μg/ml), cefotaxime (0.25 to 64 μg/ml), and cefotaxime plus clavulanic acid (0.25/4 to 64/4 μg/ml). It should be emphasized that both ceftazidime and cefotaxime should be used. Broth microdilution is performed using standard methods. Phenotypic confirmation is considered as a ≥3-twofold-serial-dilution decrease in MIC of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone (Paterson and Bonomo, 2005).

Hadziyannis evaluated a set of 12 isolates with well characterized β-lactamases using this method. Five isolates with known ESBLs and five without ESBLs were correctly identified. There was one false-positive, a *K. pneumoniae* isolate lacking ESBLs but hyper producing SHV-1 with a

ceftazidime MIC of 4 μg/ml reducing to ≤ 0.025 μg/ml with ceftazidime /clavulanic acid. One *K. oxytoca* isolate which hyper produced the chromosomal K1 β-lactamase and was resistant to aztreonam was negative for ESBL production using the criteria above (Paterson and Bonomo, 2005).

iii) Double disc confirmatory test the double disc synergy (Disc approximation test) was carried on the resistant isolates. Amoxicillin /clavulanic acid (20ug/10ug) disc was placed in the center of the Muller-Hinton agar plate on which a lawn culture of the test organism (turbidity were matched to 0.5 Mc-Farland turbidity) was prepared; ceftazidime (CAZ 30ug) and cefotaxime (CTX 30ug) were placed on either side at a distance of 15 mm center to center from the amoxicillin/calvulanic acid (AMC). Plates were incubated at 37 ˚C for 18 -24 hrs and the pattern of zone inhibition was noted. Isolates that exhibited a distinct potentiating towards amoxicillin/calvulanic acid disc were considered potential ESBL producer (Paterson and Bonomo, 2005).

2.5.3. Commercially available methods for ESBL detection

Etest for ESBLs, Vitek ESBL cards, MicroScan panels and BD Phoenix Automated Microbiology System.

2.5.4. Implications of positive phenotypic confirmatory tests

According to CLSI guidelines, isolates which have a positive phenotypic confirmatory test should be reported as resistant to all cephalosporins (except the cephamycins, cefoxitin, and cefotetan) and aztreonam,

regardless of the MIC of that particular cephalosporin (Paterson and Bonomo, 2005).

2.5.5. Quality control

When performing screening and phenotypic confirmatory tests quality control recommendations are that simultaneous testing with a non ESBL producing organism (*Escherichia coli* ATCC 25922) and an ESBL producing organism (*Klebsiella pneumoniae* ATCC 700603) also be performed. Control limits for these organisms are widely available (Paterson and Bonomo, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design

3.1.1. Type of study

This was a cross-sectional study.

3.1.2. Study area

This study was conducted in El Hawata rural hospital in Al Gadarif State. The practical part of this study was carried out in Medical Microbiology Laboratory of El Shaheed Charity Center.

3.1.3. Study duration

The study was done during the period from March to October 2019.

3.1.4. Sample size

One hundred urine and twenty wound swab specimens were collected**.**

3.1.5. Ethical consideration

A verbal consent was obtained from each patient participated in this study after informed about the value of this study.

3.2. Experimental work

3.2.1. Collection of specimens

Different types of specimens were collected. These include: urine (100) and wound swab (20).

3.2.2. Culture

Different types of culture media including MaCconkey agar, Mannitol Salt agar and Cysteine Lactose Electrolyte Deficient (CLED) (Appendix 1) were used to provide a pure isolates. The urine specimens were inoculated using a wire loop felled drop of mid-stream urine was placed on the plates of CLED and MaCconkey agar and then distributed on the surface of plate to obtain a single colonies and incubated at 37˚C for (18 _ 24hrs). After incubation the pure isolated colonies were tested for colonial morphology. The wound swabs were cultured on blood agar (Appendix 2), MaCconkey agar (Appendix 3) and Mannitol Salt agar (Appendix 4), incubated at 37°C for (18 _ 24hrs). After incubation the pure isolated colonies were tested for colonial morphology.

3.2.3. Identification

3.2.4 Gram stain

Dried smear was prepared by emulsifying a small portion of single colony on clean and dry slide that contain sterile normal saline. Then was fixed, cover smear with crystal violet stain (Appendix 5) for 30–60 seconds, rapidly washed off the stain with clean water all the water were tipped off, and the smear was covered with Lugol's iodine (Appendix6) for 30–60 seconds, washed off with clean was water, then decolorized rapidly (few seconds) with acetone–alcohol (Appendix 7), then washed immediately with clean water, the smear was covered with safranin stain

(Appendix 8) for 2 minutes, washed off with clean water, the back of the slide clean was wiped, and placed on a draining rack for the smear to air-dry, smear was examined microscopically, first with the 40 objective to check the staining and to see the distribution of material, and then with the oil immersion objective to report the bacteria and cells (Cheesbrough, 2006).

3.2.5. Biochemical tests

3.2.6. Oxidase test

A piece of filter paper was placed in a clean petridish and 2 or 3 drops were added of freshly prepared oxidase reagent (Appendix 9), using a piece of stick (not an oxidized wire loop), a colony of the test organism was removed and smear it on the filter paper and looked for the development of a blue-purple color within a few seconds. Result blue-purple color positive oxidase test (within 10 seconds) and negative oxidase test no blue-purple color (within 10 seconds) (Cheesbrough, 2006).

3.2.7. Sugars fermentation, H_2S and gas production

This is a multi tests carried out in Kligler Iron Agar (KIA) medium. They performed by inoculating KIA medium. A straight wire was used to inoculate KIA medium (Appendix 10), first the butt was stabbed and then the slope was streaked in a zigzag pattern, after inoculation (make sure the tube tops are left loose). KIA reactions are based on the fermentation

of lactose and glucose (dextrose) and the production of hydrogen sulphide. A yellow butt (acid production) and red-pink slope indicates the fermentation of glucose only, the slope is pink-red due to a reversion of the acid reaction under aerobic conditions, cracks and bubbles in the medium indicate gas production from glucose fermentation. A yellow slope and a yellow butt indicate the fermentation of lactose and possibly glucose, a red-pink slope and butt indicate no fermentation of glucose or lactose and blackening along the stab line or throughout the medium indicates hydrogen sulphide (H_2S) production (Cheesbrough, 2006).

3.2.8. Indole test

The organism was inoculated in a Bijou bottle containing 3 ml of sterile tryptone water (Appendix 11), then incubated at 35–37°C for up to 48 hs, indole was tested by adding 0.5 ml of Kovac's reagent (Appendix 12). Then Shaked gently and examined for a red ring in the surface layer within 10 minutes. Red surface layer indicate positive indole test and no red surface layer indicate negative indole test (Cheesbrough, 2006).

3.2.9. Urease test

A dense 'milky' suspension of the organism was prepared in 0.25 ml normal saline in a small tube, urease tablet was added (Appendix 13) then the tube was closed and incubated at 35–37°C for up to 4 hours or overnight. Red/purple color indicates positive urease test while yellow/orange color indicates negative urease test (Cheesbrough, 2006).

3.2.10. Citrate Utilization Test

A dense bacterial suspension was prepared in 0.25 ml sterile normal saline in small tube, citrate tablet was added (Appendix 14) and the tube was stoppered and incubated overnight at 35–37°C. Positive citrate test is indicated by red color while yellow-orange color, indicates a negative citrate test (Cheesbrough, 2006).

3.2.11. Antimicrobial susceptibility testing

After isolation the organisms were examined; to determine their susceptibility to some antibiotics which included (imipenem, ceftazidime, nitrofurantion and ciprofloxacin). Muller-Hinton agar (Appendix 15) was prepared. Commercially provided discs of antibiotics were used. Preparation of suspension turbidity was matched to 0.5 Mc-Farland turbidity and then the surface of the Petri-dish was seeded by the isolate using a disposable cotton swab. Different discs of antibiotics were placed. Incubate the plates were incubated for (18 -24 hrs) at a 37° C. After incubation, zone of inhibition were read and compared to provide chart (Cheesbrough, 2006).

3.2.12. Double disc confirmatory test

The double disc synergy (Disc approximation test) was carried on the resistant isolates. Amoxicillin/clavulanic acid (20ug/10ug) disc was placed on the center of the Muller-Hinton agar plate on which a lawn culture of the test organism (turbidity were matched to 0.5 Mc-Farland

turbidity) was prepared; ceftazidime (CAZ 30ug) and cefotaxime (CTX 30ug) were placed on either side at a distance of 15 mm center to center from the amoxicillin/calvulanic acid (AMC). Plates were incubated at 37 ˚C for 18 -24 hrs and the pattern of zone inhibition was noted. Isolates that exhibited a distinct potentiating towards amoxicillin/calvulanic acid disc were considered potential ESBL producers.

3.3. Data analysis

The data were analyzed by using the statistical packaged for social science (SPSS) version 16 by using chi-square test to determine the significance of data compared to the ($P.value\ as \leq .05$) was considered to be statistically significant.

CHAPTER FOUR

RESULTS

A total of 120 clinical specimens were collected from El Hawata Rural Hospital. The specimens were collected from both males (59=49%) and females (61=51%) (Table 1). The specimens included 100 urine and 20 wound swabs as showed in table 2.

The results revealed different bacteria isolates. These were (65=65%) *E. coli*, (15=15%) *K. pneuomniae* (10=10%) *Ps. aeruginosa* and (10=10%) *S. aureus.* 60 isolates of *E. coli* were obtained from the urine specimens and 5 from wound swabs, 10 isolates of *K. pneumoniae* were obtained from urine specimens and 5 from wound swabs, 8 isolate of *Ps. aeruginosa* were obtained from urine specimens and 2 from wound swabs, 2 isolates of *S. aureus* were obtained from urine and 8 from the wound swabs as illustrated in table 3 and table 4.

The isolates were (100%) sensitive to imipenem, (83%) sensitive to ciprofloxacin, (79%) sensitive to nitrofurantoin and (48%) sensitive to ceftazidime as explained in table 5.

41% of the isolates were positive to ESBL production. The isolates showed varied positive results as 10 out of 15 *K. pneumoniae* (67%) were positive, 6 out of 10 *Ps. aeurginosa* (60%) were positive, 23 out of 65 *E. coli* (35%) isolates were identified as positive for ESBL production, and 2 of 10 isolates of *S. aureus* (20%) were positive as showed in table 6.

The ESBL producing bacteria showed an insignificant relation with the gender as showed in table 7.

Table 1. Distribution of clinical specimens according to the gender

Specimens	**Males**	**Females**	**Total**
Urine	45	55	100
Wound swab	14	6	20
Total	59	61	120

Table 2. Frequency of different clinical specimens

Specimen	**Total**
Urine	100
Wound swab	20

Table 3. Frequency of different clinical isolates

Isolate	**Frequency (%)**
No growth	20(17%)
E. coli	65(54%)
K. pneumoniae	15(13%)
Ps. aeruginosa	10(8%)
S. aureus	10(8%)
Total	120(100%)

Table 4. Distribution of bacterial isolates among the clinical specimens

Isolate	Urine	Wound swab	Total
E. coli	60	5	65
K. pneuomniae	10	5	15
Ps. aeruginosa	8	2	10
S. aureus	2	8	10
Total	80	20	100

Table 5. Susceptibility to the antibiotic discs among different clinical isolates

	Imp		**Caz**		**Nit**		**Cip**		
Isolate	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**Total**
E. coli	65	0	21	44	56	9	55	10	65
k. pneumoniae	15	0	10	5	11	4	9	6	15
Ps. aeruginosa	10	0	8	2	2	8	6	4	10
S. aureus	10	0	9	1	10	0	8	2	10
Total	100	0	48	52	79	21	78	22	100

Table 6. Distribution of ESBL production among different clinical isolates

Isolate	ESBL positive	ESBL negative	Total
K. pneuomniae	10(67%)	5(33%)	15
Ps. aeruginosa	6(60%)	4(40%)	10
E. coli	23(35%)	42(65%)	65
S. aureus	2(20%)	8(80%)	10
Total	41(41%)	59(59%)	100

Table 7. Distribution of ESBL production according to gender

Gender	**Positive**	**Negative**	**Total**
Males	20	39	49
Females	21	30	51
Total	41	59	100

(*P. value*= 0.53)

CHAPTER FIVE

DISCUSSION

5.1. Discussion

The resistance to the third generation cephalosporins has become a cause of concern especially among Enterobactericaeae. Members of this family cause non nosocomial infection. The frequency of ESBL producing bacteria among the members of Enterobactericaeae constitute a serious threat to current β-lactam antibiotics therapy leading to treatment failure. Among the wide array of antibiotics, β-lactam antibiotics are the most widely used for more than (50%) of all the systemic infections (Kumar *et al.,* 2006).

The most effective antibiotic against ESBLs producing bacteria was found to be imipenem, which yield a total coverage of all isolated bacteria in this study. This result disagrees with results of Mirbagheri *et al.,* 2015 which revealed that 63% resistance to imipenem. This is because there in a limited use of imipenem in El Hawata.

This study demonstrated the presence of ESBL mediated resistance in clinical isolates of patients in El Hawata Rural Hospital in Al Gadarif State. The frequency of ESBL producing isolates was found to be (41%). This figure was high compared to the findings (28.2%) reported in Tanzania (Nduglile *et al.,* 2005), but lower than the finding (53%) in the study

carried out in the Khartoum State (Mekki *et al.,* 2010), but much higher than the (6.5%) reported in Saudi Arabia (Kader and Kumar, 2004), but much lower than the (60.9%) observed in Egypt (Mohamed-algamy *et al.,* 2004) and (78%) in Pakistan (Hussain *et al.,* 2011).

The ESBL production is found to be higher in *E. coli* (23 of 41= 55%) followed by *K. pneuomniae* (10 of 41= 24%) then *Ps. aeruginosa* (6 of 41= 14%) and less frequent in *S. aureus* (2 of 41= 5%). This result agreed with Nepal *et al.* in 2017 that revealed 268 (17.1%) of samples were positive for production of ESBL. Among which, *E. coli* and *K. pneumoniae* were isolated from 138 (51.5%) and 39 (14.6%) samples respectively.

Most of the ESBL producing bacteria were isolated from urine specimens 30 of 41(73%). This matched with the study of Al-Garni *et al.* in 2018 that revealed the highest production of ESBL was found in urine (62%).

There was no significanct difference in ESBL production among gender. That conflicted with the study of Al-Garni *et al.* in 2018 that revealed the ESBL producing organisms were mainly isolated from the females of medical ward.

Over all these findings indicate that the frequency of ESBL producers is world-wide varied. In this study there was a significant difference observed among different groups. This study showed insignificant difference in the production of ESBL as found with the study of (Akanbi *et al.,* 2013).

5.2. Conclusion

The study showed a high frequency of ESBL production among *K. pneuomniae* and *Ps. aeruginosa* from the different clinical specimens.

5.3. Recommendations

1- Culture and susceptibility testing must be performed to determine the appropriate antibiotic therapy.

2- Performing the polymerase chain reaction technique to determine the responsible gene for ESBL production and increase the sample size.

3- Continuous studies about the antibiotic resistance because of rapid bacterial resistance evolution.

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APPENDICES

Appendix (1): CLED Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Peptone	4.00
Lactose	10.00
Meats extract	3.00
L-Cystine	0.128
Agar	15.00
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 36.1 Grams in 998 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

Appendix (2): Blood Agar Base (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Tryptose	10.00
HM peptone B	10.00
Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 40 Grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

Appendix (3): MacConkey Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Peptone (meat and casein)	3.00
Pancreatic digest of gelatin	17.00
Lactose monohydrate	10.00
Bile salts	1.500
Sodium chloride	5.50
Crystal violet	0.001
Neutral red	0.030
Agar	13.50
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 49.53 Grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

Appendix (5): Crystal violet (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Crystal violet	20 g
Ammonium oxalate	9 g
Ethanol or methanol, absolute	95 ml

Preparation

Weigh the crystal violet on a piece of clean paper (pre weighed), transfer to a brown bottle pre marked to hold 1 liter, add the absolute ethanol or methanol (technical grade is suitable) and mix until the dye is completely dissolved, weight the ammonium oxalate and dissolve in about 200 ml of distilled water, add to the stain, make up to the 1 liter mark with distilled water, and mix well (Caution: Ammonium oxalate is a toxic chemical, therefore handle it with care), label the bottle, and store it at room temperature. The stain is stable for several months.

Appendix (6): Lugol's iodine (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Potassium iodide	20 g
Iodine	10 g

Preparation

Weigh the potassium iodide, and transfer to a brown bottle pre marked to hold 1 liter, add about a quarter of the volume of water, and mix until the potassium iodide is completely dissolved, weight the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved (Caution: Iodine is injurious to health if inhaled or allowed to come in contact with the eyes, therefore handle it with care in a well ventilated room and make up to the 1 liter mark with distilled water, and mix well. Label the bottle, and mark it Toxic. Store it in a dark place at room temperature. Renew the solution if its color fades.

Appendix (7): Acetone-alcohol decolorizer (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Acetone	500 ml
Ethanol or methanol, absolute	475 ml

Preparation

Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methyl alcohol), transfer the solution to a screw-cap bottle of 1 liter capacity, technical grade is adequate, measure the acetone, and add immediately to the alcohol solution mix well (Caution: Acetone is a highly flammable chemical that vaporizes rapidly, therefore use it well away from an open flame) and label the bottle, and mark it Highly

Flammable. Store in a safe place at room temperature. The reagent is stable indefinitely.

Appendix (8): Safranin (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Safranin O	0.50
Ethyl alcohol, 95%	100.00

Appendix (9): Oxidase reagent (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Tetramethyl-p-phenylenediaminedihydrochloride	0.1 g

Preparation

Dissolve the chemical in the water. The reagent is not stable. It is therefore best prepared immediately before use.

Appendix (10): Kligler Iron Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Peptic digest of animal tissue	15.00
Beef extract	3.00
Yeast extract	3.00
Protease peptone	5.00
Lactose	10.00
Dextrose	1.00

Ferrous sulphate	0.20
Sodium chloride	5.00
Sodium trisulphate	0.30
Phenol red	0.024
Agar	15.00
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 57.52 Grams in 1000 ml distilled water. Heat to boil to dissolve the medium completely. Mix well and distribute into tubes. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes. Cool the tubes tis etas slopes with 1 inch butts.

Appendix (11): Peptone Water (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Peptic digest of animal tissue	10.00
Sodium chloride	5.00
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 15.0 Grams in 100 ml distilled water. Mix well and dispense into tubes with or without inverted Durham's tubes and sterilize by autoclaving at 15Ibs pressure (121 °C) for 15 minutes.

Appendix (12): Kovac's Reagent (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
p-dimethyl aminobenzaldehyde	10 g
isoamyl alcohol	150 ml
Concentrated hydrochloric acid	50 ml

Preparation

Kovac's reagent is prepared by dissolving 10 gm of p-dimethyl aminobenzaldehyde in 150 ml of isoamyl alcohol and then slowly adding 50 ml of concentrated hydrochloric acid.

Appendix (13): Urea Agar (Christensen) (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium chloride	5.00
Dipotassiumphpsphate	1.20
Monopotasiumphosphate	0.80
Phenol red	0.012
Agar	15.00
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 24 Grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 Ibs pressure (121 °C) for 15 minutes. Cool to 50°C and aseptically add 50 ml of sterile 40% urea solution and mix well. Dispense into sterile tubes and allow to set on slanting position. Don't over heat or reheat the medium as urea decomposes very easily.

Appendix (14): Simmons Citrate Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.00
Dipotassiumphpsphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromothymol blue	0.08
Agar	15.00
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 24.28Grams in 100 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired in tubes or flasks. sterilize by autoclaving at 15 Ibs pressure (121 °C) for 15 minutes.

Appendix (15): Muller-Hinton Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients	g/L
HM infusion B from Beef	300.0
Sodium chloride	17.50
Starch	1.50
Agar	15.00
Final pH (at 25°C)	7.3±0.2

Preparation

Suspend 38 Grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.