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Detection of Extended–spectrum Beta Lactamase Genes in Urinary Tract Isolated Bacteria among Pregnant Women

الكشف عن جينات إنزيمات البيتالاكتام ممتدة الطيف في البكتيريا المعزوله من الجهاز البولي لدي الحوامل

A Dissertation Submitted in Partial Fulfillment for the Requirements of M.Sc Degree in Medical Laboratory Science (Microbiology)

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ ۗ وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ أَنْ يُقْضَىٰ إِلَيْكَ وَحْيُهُ ۖ وَقُلْ رَبِّ زِدْنِي عِلْمًا ﴾

سورة طه الاية(114)

Dedication

To my mother, soul of my honorable father, brothers, sisters, friends, colleagues and teachers…

ACKNOWELGDEMENTS

First of all thanks to ALMIGHTY ALLAH who gives me the power to complete this work.

With a great deal of respect I would like to express my thanks to my supervisor Dr. Yousif FadAllah Hamed Elnil for his advice, patience, encouragement and valuable supervision during this study. Also my thanks and gratitude extended to Miss Suhair Ramadan for her great help and all my colleagues who helped me throughout this study.

ABSTRACT

The aim of this study was to detect extended spectrum β-lactamase (ESBL) in bacterial isolated from urine collected from urinary tract infected pregnant women. A total of 100 midstream urine samples were collected from Omdurman Military Hospital from April to May 2013. Specimens were cultured on 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 *Klebsiella Oxytoca* 15 (26.8%) and *Escherichia coli* 10(17.9%),while the most abundant Gram positive was *Staphylococcus Saprophyticus.* In The susceptibility test, 5 isolates were resistant to 3rd generation cephalosporin (ceftazidime, ceftriaone and cefotaxime) as follow: 2 *K. pneumoniae* ,2 *E. col*i and 1 *Proteus 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. The PCR was done for 5 isolates using TEM, SHV, and CTX-M primers, TEM gene was the most predominant one followed by CTX-M 3and the least one was SHV gene. From the results it could be Tconcluded that these genes are responsible for the resistance of cephalosporin.

المستخلص

الهدف من هذه الدراسه الكشف عن انزيمات البيتالاكتام الممتدة الطيف في البكتريا المعزوله من البول, جمعت من النساء الحوامل يشتبه في التهاب المسالك البوليه. تم جمع 100 عينة بول من مستشفي أم درمان العسكري خلال شهر ابريل, تم تزريع علي أجار لتحديد البكتريا المسببة التهاب المسالك البولية بواسطة الشكل الظاهري للمستعمره, صبغة جرام واختبارات الكيمياء الحيوية من اصل 100 عينة بول؛أعطي 56 (56%) نموا عالي وكانت منه عصيات سلبية الجرام 42 (75%) و 14 (25%) ايجابية الجرام,والاكثر تردد من سلبية الجرام هي الكلبسيلة الاكسوتوكيه 15 (26.8%), والاشريكية القولونية 10 (17.9%) والاكثر تردد من ايجابية الجرام العنقوديه سابروفيتيكس 13 (23.2%) . اختبار الحساسية 5 بكتريا معزوله مقاومة للجيل الثالث سيفالوسبورين (السيفتازيديم, السيفتريكسون و السيفوتاكزيم) 2 الكلبسيلة الرئويه, 2 الاشريكية القولونيه و 1 البروتيس فولقاريس. اختبار انزيم البيتالاكتام الممتدة الطيف تم لكل البكتريا المقاومه للجيل الثالث سيفالوسبورين و اكد عن طريق استخدام الغرص المزدوج التأزرخمسه من البكتريا المعزوله المقاومه اعطت نتيجه ايجابيه 5 (11.9%), وقد تم التفاعل الانزيمي المتسلسل للخمسه البكتريات المقاومه عن طريق استخدام الجين TEM,SHV. CTX

من النتائج يمكن ان نخلص ال ان هذه الجينات هي المسؤله . SHVواقل واحد CTX-Mويليليه TEMوالجين الاكثر شيوعا عن مقاومة السيفالوسبورينز

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List of abbreviation

AmpC: class C β-lactamases

ATCC: American Type Culture Collection

CTX-M: Cefotaximase

DDST: Double Disc Synergy Test

ESBL: Extended-spectrum β-lactamase

MIC: minimum inhibitory concentration

PCR: polymerase chain reaction

SHV: Sulfhydryl variable

SPP : Species

TEM: β-lactamase named after a Greek patient Temoneira

UTI : urinary tract infection

CHAPTER ONE

1. INTRODUCTION

1.1 Introduction

Extended spectrum beta lactamases(ESBL) producing organisms are emerging pathogens with greatest spread in general population, and their presence in clinical infection can result in treatment failure when using 3rd generation cephalosporins (Pasteron and Bonomo, 2005). The extended spectrum β-lactamases are enzymes elaborated by some bacteria and responsible for their resistance to extended spectrum 3rd generation cephalosporins e.g. ceftazidim, cefotaxime ceftriaxone and monobactam e.g. azteronem (Carlos , 2000).These enzymes catalyze the hydrolysis of the β-lactam ring of antibiotics, ESBLs have been reported worldwide in many different genera of Enterobactericeae, Pseudomonas aeroginosa, (Friedman et al., 2008). *Klebsiella pneumonia* and E. coli (Agrawal et al., 2008). ESBL producing organisms are often resistant to several other classes of antibiotics, as the plasmids with the gene encoding ESBLs often carry other resistance determinants, initially ESBL producing organisms were isolated from community (Pitout and Laupland, 2008).The ESBLs are mediated by bla TEM, bla SHV and bla CTX-M genes in *Enterobacteriaceae* and other Gram- negative bacteria, numerus molecular typing methods have been developed for their identification. The multiplex PCR assay allowed the identification of bla TEM, bla SHV, and bla CTX-M genes in aseries of clinical isolates of *Enterobacteriaceae* with previously characterized ESBL phenotype (Monstein et al., 2007). The first plasmid β-lactamase TEM1 was described in Germany 1980, and in France 1985 (Perez et al., 2007).The National Committee for Clinical Laboratory Standards (NCCLS) recommended that microbiology laboratories reported ESBL –producing isolates of *E.coli* and *Klebsiella* species are resistant to all penicillins, cephalosporins and azteronam, irrespective of their individual in vitro test result.Urinary tract infections (UTI) that affect parts of the urinary system. Occur more in women than men, especially in pregnancy due to the increased risk of kidney infection. During pregnancy high progesterone level elevated the risk of decreased muscle tone of the ureters and bladder which lead to greater likelihood of reflux where urine flows back up the ureters and towards the kidney (Dielubanzaand Schaeffer, 2011). The bacterial spectrum and antimicrobial resistance may vary temporarily and geographically, each institution must undertake its own local evaluation such an evaluation may also be useful to detect emerging trends of antimicrobial resistance (Wagenlehner et al., 2008).

The objectives of this study were to: Isolate and identify the important bacterial pathogen causing UTI in pregnant women. Determine the drug resistance patterns of the isolated bacteria with emphasis to third generation cephalosporins , and use molecular biology tools e.g. PCR in the detection of ESBL encoding genes

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Extended spectrum beta lactamase (ESBL)

2.1.1. History of ESBL:

Members of the family Enterobacteriaceae commonly express plasmid encoded β-lactamases e.g.: (TEM1, TEM2, SHV and CTX-M). Which confers resistance to penicillin but not to expanded spectrum cephalosporin; in the mid 1980 a new group of extended spectrum β-lactamases (ESBL) were detected.

First detection was in GERMANY in 1983 (Knoth *et al.*, 2003)

2.1.2. Classification:

2.2.1 Functional classification

Was described by (Bush *et al*., 1995) as different groups

Group 1

CEPHALOSPORINASE, Molecular Class C *(not inhibited by clavulanic acid)*

Group 1 are cephalosporinases not inhibited by clavulinic acid , belonging to the molecular class C

Group 2

Group 2 are penicillinases, cephalosporinases, or both inhibited by clavulanic acid, corresponding to the molecular classes A and D reflecting the original TEM and SHV genes. However, because of the increasing number of TEM- and SHV-derived β-lactamases, they were divided into two subclasses, 2a and 2b

GROUP 2a

PENICILLINASE, Molecular Class A

The *2a subgroup* contains just penicillinases.

GROUP 2b

BROAD-SPECTRUM, Molecular Class A

2b opposite to 2a , 2b are broad-spectrum β-lactamases, meaning that they are capable of inactivating penicillins and cephalosporins at the same rate. Furthermore, new subgroups were segregated from subgroup 2b:

GROUP 2be

Extended spectrum Class A

Subgroup 2be, with the letter "e" for Extended spectrum of activity, represents the ESBLs, which are capable of inactivating third-generation cephalosporins (ceftazidime, cefotaxime, and cefpodoxime) as well as monobactams (aztreonam).

GROUP 2br

INHIBITOR-RESISTANT, Molecular Class A *(diminished inhibition by clavulanic acid)*

The *2br* enzymes, with the letter "r" denoting reduced binding to clavulanic acid and sulbactam, are also called inhibitor-resistant TEM-derivative enzymes; nevertheless, they are commonly still susceptible to tazobactam, except where an amino acid replacement exists at position met69.

GROUP 2c

more CARBENICILLINASE, Molecular Class A

Later*subgroup 2c* was segregated from group 2 because these enzymes inactivate carbenicillin more than benzylpenicillin, with some effect on cloxacillin.

GROUP 2d

CLOXACILANASE, Molecular Class D or A

Subgroup 2d enzymes inactivate cloxacillin than benzylpenicillin, with some activity against carbenicillin; these enzymes are poorly inhibited by clavulanic acid, and some of them are ESBLs

the correct term is "OXACILLINASE". These enzymes are able to inactivate the oxazolylpenicillins like oxacillin, cloxacillin, dicloxacillin. The enzymes belong to the molecular class D not molecular class A.

GROUP 2e

CEPHALOSPORINASE, Molecular Class A

Subgroup 2e enzymes are cephalosporinases that can also hydrolyse monobactams, and they are inhibited by clavulanic acid.

GROUP 2f

CARBAPENAMASE olecular Class A

Subgroup 2f was added because these are serine-based carbapenemases, in contrast to the zinc-based carbapenemases included in group 3

Group 3

METALLOENZYME, Molecular Class B *(not inhibited by clavulanic acid)*

Group 3 are the zinc-based or metallo beta-lactamases, corresponding to the molecular class B, which are the only enzymes acting by the metal ion zinc, as discussed above. Metallo B-lactamases are able to hydrolyse penicillins, cephalosporins, and carbapenems. Thus, carbapenems are inhibited by both group 2f (serine-based mechanism) and group 3 (zinc-based mechanism)

Group 4

PENICILLINASE, No Molecular Class *(not inhibited by clavulanic acid)*

Group 4 are penicillinases that are not inhibited by clavulanic acid, and they do not yet have a corresponding molecular class.

Molecular classification

Renamed Beta-lactamase when the structure of the Beta-lactam ring was finally elucidated The molecular classification of β-lactamases is based on the nucleotide

and amino acid sequences in these enzymes. To date, four classes are recognised (A-D), correlating with the functional classification. Classes A, C, and D act by a serine-based mechanism, whereas class B or metallo-β-lactamases need zinc for their action.

2.1.3. Types

TEM beta-lactamases (class A)

TEM-1 is the most commonly encountered beta-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in E. coli is due to the production of TEM-1. Also responsible for the ampicillin and penicillin resistance that is seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers. Although TEM-type beta-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found in other species of Gram-negative bacteria with increasing frequency. The amino acid substitutions responsible for the ESBL phenotype cluster around the active site of the enzyme and change its configuration, allowing access to oxyimino-beta-lactam substrates. Opening the active site to beta-lactam substrates also typically enhances the susceptibility of the enzyme to b-lactamase inhibitors, such as clavulanic acid. Single amino acid substitutions at positions 104, 164, 238, and 240 produce the ESBL phenotype, but ESBLs with the broadest spectrum usually have more than a single amino acid substitution. Based upon different combinations of changes, currently 140 TEM-type enzymes have been described (Paterson *et al* ., 2003).

(SHV beta-lactamases class A)

SHV-1 shares 68 percent of its amino acids with TEM-1 and has a similar overall structure for up to 20% of the plasmid-ampcillin resistance in this species ESBLs in the SHV-1 beta-lactamase is most commonly found in

K. pneumoniae and is responsible this family also have amino acid changes around the active site, most commonly at positions 238 or 238 and 240. More than 60 SHV varieties are known. They are the predominant ESBL type in Europe and the United States and are found worldwide. SHV-5 and SHV-12 are among the most common (Paterson *et al* ., 2003).

CTX-M beta-lactamases (Class A)

These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e.g., ceftazidime, ceftriaxone, or cefepime). Rather than arising by mutation, they represent examples of plasmid acquisition of beta-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms. These enzymes are not very closely related to TEM or SHV beta-lactamases in that they show only approximately 40% identity with these two commonly isolated beta-lactamases. More than 80 CTX-M enzymes are currently known. Despite their name, a few are more active on ceftazidime than cefotaxime. They have mainly been found in strains of *Salmonella enterica* serovar *Typhimurium* and *E. coli*, but have also been described in other species of Enterobacteriaceae and are the predominant ESBL type in part of south America .They are also seen in Eastern Europe ,*CTX-M-14*,*CTX-M-3* and *CTX-M-2* are the most widespread. *CTX-M-15* is currently the most widespread type in *E. coli* the UK and is widely prevalent in the community (Woodford *et al.*,2006).

OXA beta-lactamases (Class D)

OXA beta-lactamases were long recognized as a less common but also plasmid-mediated beta-lactamase variety that could hydrolyze oxacillin and related anti-staphylococcal penicillins. These beta-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d. The

OXA-type beta-lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid. Amino acid substitutions in OXA enzymes can also give the ESBL phenotype. While most ESBLs have been found in *E. coli*, *K. pneumoniae*, and other Enterobacteriaceae, the OXA-type ESBLs have been found mainly in *P. aeruginosa*. OXA-type ESBLs have been found mainly in *Pseudomonas aeruginosa* isolates from Turkey and France. The OXA beta-lactamase family was originally created as a phenotypic rather than a genotypic group for a few beta-lactamases that had a specific hydrolysis profile. Therefore, there is as little as 20% sequence homology among some of the members of this family. However, recent additions to this family show some degree of homology to one or more of the existing members of the OXA beta-lactamase family. Some confer resistance predominantly to ceftazidime, but OXA-17 confers greater resistance to cefotaxime and cefepime than it does resistance to ceftazidime (Woodford *et al.*,2006)

Others Types of ESBL genes

Other plasmid-mediated ESBLs, such as PER, VEB, GES, and IBC beta-lactamases, have been described but are uncommon and have been found mainly in *P. 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 South East Asia; and GES-1, GES-2 and IBC-2 in isolates from South Africa, France and Greece. PER-1 is also common in multiresistant acinetobacter species in Korea and Turkey. Some of these enzymes are found in Enterobacteriaceae as well, whereas other uncommon ESBLs (such as BES-1, IBC-1, SFO-1 and TLA-1) have been found only in Enterobacteriaceae.

2.2. Urinary tract infections (UTIs):

There are two types of UTIs: lower and upper. Lower UTIs occur in urethra (urethritis) or bladder (cystitis). Upper UTIs are infections that involve kidneys (pyelonephritis), or ureters (ureteritis), or both. Upper UTIs can occur in both men and women as a complication of lower UTI. Complicated UTIs are resulting from anatomic obstructions of the urinary tract or catheterization. These abnormalities increase the volume of residual urine and interfere with the normal clearance of bacteria by urination. Such factors include prostate enlargement, sagging uterus and expansion of the uterus during pregnancy, paraplegia, spinabifida, scar tissue formation and catheterization. Uncomplicated acute UTIs refer to that seen in patients with normal anatomic structure and function of the urinary tract (Najar *et al*., 2009: Foxman, 2010).

2.2.1 Symptoms of urinary tract infection

Urethritis appears as discomfort during urination. Most of the cases of purulent urethritis without cystitis are sexually transmitted infection is limited to the urethra. Hemorrhagic cystitis is characterized by large quantities of visible blood in the urine. It can be caused by an infection (bacterial) or as a result of radiation, cancer chemotherapy or select immunosuppressive medications (Silva *et al*., 2010).

Pyelonephritis symptoms include fever, back pain, costovertebral angle tenderness, nausea and vomiting and possibility of urinary urgency and frequency urine contains white blood cell casts composed of cells that were tightly packed in the tubules and excreted in proteinaceous matrix (lee *et al*., 2009).

2.2.2 Causative organisms of urinary tract infections:

The vast majority of UTIs are due to patients own fecal bacteria. Approximately 80% of acute uncomplicated UTIs are caused by *E. coli*, 10 to 20 % are caused by coagulase – negative *Staphylococcus saprophyticus* and 5% or less are caused by

other Enterobacteriaceae such as *Proteus* and *Klebsiella* or by Enterococci species (Nicolettia *et al*., 2010).

The most common causes of complicated UTIs are *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter Cloacae*, *Serratia marcescens*, *Pseudomonas aeruginosa* and Gram-positive organism such as Enterococci, coagulase–negative Staphylococci and *Staphylococcus aureus* (Bader *et al*., 2010; Pallett and Hand, 2010).

2.2.3 Diagnosis of urinary tract infection

The diagnosis based on clinical symptoms and the clinical diagnosis includes history and physical examination.

Microscopical examination of urine

UTI can readily be diagnosed by microscopical examination of urine. Standardized centrifuge urinary sediment investigated under a cover slip is recommended as the routine procedure because it is cheap and the differentiation of formed element (red, white cell and bacteria) is easier in thin fluid layers than in traditional glass chambers. Centrifugation always leads to loss of particles and may produce inaccurate result in quantitative terms. On the other hand, in un spun samples a number of relevant elements can be missed. Thus the results after centrifugation with standardized procedures are more sensitive and specific. When compared with bright-field microscopy, the phase-contrast technique allows better detection of most elements, especially of bacteria. The counts are usually given per low-power field or high-power field. At the high magnification (40x) the presence of >10 white blood cell/high-power field is indicative of pyuria (Haider *et al*., 2010)

Urine culture

The diagnosis of UTI is confirmed by culturing the organism from urine. Most bacteria that cause urinary infection grow readily and the clinical diagnosis of UTI

is usually confirmed within 24hours. Patients suspected of UTI are usually asked to collect a mid-stream sample after cleaning the perineum or glans penis with soap and water. An early-morning collection is best because the concentration of bacteria in the urine is a greatest prior to the morning voiding. The urine is then refrigerated or taken to the laboratory for immediate culture. Urine can be stored in a refrigerator for up 24 hours without any loss of bacterial viability (Gupta *et al.*, 2011).

2.2.4 Epidemiology

From epidemiological viewpoint UTIs occur in two general setting: community-acquired and hospital (nosocomially) acquired, *E. coli* caused about 80% of community-acquired UTI so it represent the most common cause of UTI (Mims *et al* ., 2004).

2.3. Typical characteristics of Enterobacteriaceae

Is the largest, most heterogeneous collection of medically important Gram-negative bacilli. Total of 32 genera and more than 130 species have been described, these genera have been classified based on biochemical properties, antigenic structure, nucleic acid hybridization and sequencing; are ubiquitous organisms, being found worldwide in soil, water, and vegetation, and are part of the normal intestinal flora of most animals including humans (Murray *et al*., 2002).

The wall, cell membrane and internal structures are morphologically similar for all enterobacteriaceae, the major cell wall antigen is the heat stable lipopolysaccharide LPS and it consists of three components: the somatic O polysaccharide, a core polysaccharide and lipid A. The serologic classification of the Enterobacteriaceae is based on three major groups of antigen: somatic O polysaccharide, capsular K antigen (either protein or polysaccharide) and flagellar H protein. Numerous

virulent factors have been identified in the member of the family Enterobactericeae. Some are common to all genera and others are unique to specific virulent strains (Murray *et al.*, 2002)

2.4 *Escherichia*

The genus *Escherichia* consists of five species: *E.blattae, E.fergusonii, E.hermanii, and E.vulneris. E.coli* is most numerous aerobic commensally inhabitant of the large intestine in human.

E.coli can be commonly found in animal faeces and the lower intestines of mammals, and they possess adhesive fimbriae that promote binding to intestinal, epithelial cells. *E.coli* can also be found in environments at a higher temperature, such as on the edge of hot springs in our intestines, *E.coli* is probably the most famous member of the *Enterobacteriaceae* group, since it is a model organism and lots of our knowledge of biochemical processes and genetics derive from this species (Daoud and Afif, 2011).

2.5. *Klebsiella*

The genus *Klebsiella* is among the oldest known genera in the Enterobacteriaceae described for the first time by Trevisan in 1885. *Klebsiella* consist of three species: *K. oxytoca, K. pneumoniae and K. variicola*.

Klebsiella species are non-motile and have a prominent mucoid polysaccharide based capsule (K antigen). The capsule protects from phagocytosis and aids to adherence. Due to the capsule *Klebsiella* form large moist colonies on solid culture media .*K. pneumoniae* are widely distributed in the environment where they contribute to biochemical and geochemical processes. Like *E.coli*, *Klebsiella* are colonizers of the gastrointestinal tract (Nordmann *et al.*, 2009). *Klebsiella* are

ubiquitous and may colonize the skin, pharynx, or gastrointestinal tract in humans. *K. pneumoniae* and *K. oxytoca* are both opportunistic pathogens found in the environment and on mammalian mucosal surfaces; they are commonly transferred by hands of hospital personnel (Miftode *et al* ., 2008).

2.6. Other enterobacterial genera

Other enterobacterial species most commonly encountered are *Citrobacter* ., *Hafnia alvei*, *Proteus mirabilis* , *Salmonella spp* ,*serratia spp* and *Shigella* .*Citrobacter* genus consists of twelve species and can be isolate from water, sewage soils and food as well as from the faeces of man and other animals, where they may be part of the normal microbiota of the large intestine the genus *Hafnia* contains a single species, *H. alvei*, and it is generally motile the *Yersinia* genus contains 14 species, and these are relatively slow growers among the Enterobacteriaceae. *Y.pestis, Y.pseudotuberculosis and Y.enterocolitica* are very well documented human pathogen. *Yersinia* displays their biochemical characteristics most reliably at temperatures between 25 and 32 ºC.

The *Proteus* genus is highly motile and does not form regular colonies. Instead, swarming colonies are formed when plated on a non-inhibitory media. *P. mirabilis* is considered to be the most important member of this genus. The genus *Salmonella* consists of several serotypes and most of them are motile. The *Serratia* genus contains ten species and two subspecies.

However, only two species are clinically commonly isolated. They are *S. liquefaciens* and *S. marcescens.* Often produce prodigiosin, a characteristic red pigment, when grown at 20 ºC. Most of the species are motile. *Serratia* strains can be distinguished from other enterobacterial species by their unique production of three enzymes: DNase, lipase and gelatinase. The *Shigella* genus consisting of four

species is closely related to *Escherichia* and considered to be a subspecies of *E. coli*. *Shigella* is non-motile and anaergenic; I .e .it does not produce gas from glucose (Larson *et al*., 2005; Paterson, 2006; Halstead *et al.*, 2007; Hidron *et al*., 2008) .

2.7. Gram-positive cocci

There are two medically important genera of gram-positive cocci: Streptococcus and Staphylococcus. Two of the most important human pathogens, *Staphylococcus aureus* and *Streptococcus pyogenes.* Staphylococci and Streptococci non motile and do not form spores distinguished by two main criteria microscopically, Staphylococci appear in grapelike clusters, where streptococci are in chain and biochemical, Staphylococci produce catalase (degrade hydrogen peroxide), whereas streptococci do not.

2.8. ESBL

Members of the family Enterobacteriaceae commonly express plasmid encoded β-lactamases. ESBLs are beta-lactamases that hydrolyze extended spectrum cephalosporin with oxyiminoside chain this cephalosporin include cefotaxime, ceftriaxone, and ceftazidime as well as the oxymino, monobactam and aztreonam. Thus ESBLs confirm resistance to these antibiotic and related oxymino beta-lactams. Typically, they derive from gene for TEM1, TEM2, SHV1 and CTX-M by mutation that alters the amino acid configuration around the active site of these β-lactamases. This extended the spectrum of β-lactam antibiotic susceptible to hydrolysis by these enzyme increasing number of ESBL not of TEM or SHV lineage have recently been described (Emery and Weymouth, 1997).

2.9. Detection of ESBL

2.9.1 Screening test for ESBL

2.9.1.1. Disc diffusion method

According to the CLSI guidelines, isolates showing inhibition zone size of <22mm with ceftazidime (30µg), <27mm with cefotaxime (30µg), <25mm with ceftriaxone (30µg), <27mm with aztreonam (30µg) and <22mm with cefpodoxime (10µg), was identified as potential ESBL producers and short listed for confirmation of ESBL production recently. Chromogenic designed specifically for screening and identification of ESBLs producing Enterobacteriaceae, have become commercially available (Black *et al.,* 2005).

2.9.2 Confirmatory test

2.9.2.1. Double disc synergy test (DDST)

The disc synergy test (DDST) is oldest method for phenotypic confirmation of ESBLs producing organisms, first proposed in 1980 (Jarlier *et al.,* 1988) aceftazidime 30µg disc and amoxicillin/clavulanic acid 20+10 µg disc will be placed 25-30 mm a part, center to center. Following over night incubation in aerobic at 37ºC, ESBL production is inferred when the zone of inhibition around the ceftazidime disc is expanded by the clavulanate.

2.9.2.2. Modified double disc diffusion test

Muller Hinton agar media will be inoculated with standardized inoculums (corresponding to 0.5 McFarland standard tube using sterile cotton swab. Augmentin (20mg amoxicillin and 10mg clavulanic acid AMC) disc will be placed in the center of the plate and test discs of 3rd generation cephalosporin disc will be placed at 15mm distance from the Augmentin disc. The plate will be incubated overnight at 37 ºC. ESBL production consider positive if the zone of inhibition

around the test disc increased towards the Augmentin disc or neither disc will be inhibitory alone but bacterial growth will be inhibited were the two antibiotics diffuse together .enhancement of the zone of inhibition around one or more of β-lactam containing disc towards the clavulanic acid containing disc is indicative of ESBL production. And interpretation is subjective.

2.9.2.3. Disc replacement method for ESBL confirmation

Two Amoxyclave (AMC 30mg) discs will be placed on Muller-Hinton agar inoculated with the bacterial isolate. After 1hour at room temperature, the disc were removed and replaced with ceftazidizm (RP-30) and ceftriaxone (AUF30). Each cephalosporin disc will be incubated at 37Cº for 18-24hours and read for evidence of ESBL production. Positive disc replacement method will be indicated by increased inhibition zone of 5mm and above between the inhibition zone formed by Augmentin. Replaced cephalosporin disc and those placed independently. The following two procedures will be carried out in the present study as per CLSI guideline.

2.9.3 E-Test

The E-test strip (AB Biodisk, Solna; Sweden) carries two ingredients: on the one end, ceftazidime and on the opposite end, ceftazidime plus clavullinic acid. MIC is interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge. A ratio of ceftazidime MIC to cetazidime-clavulinic acid MIC equal to or greater than 8 indicates the presence of ESBL. The reported sensitivity of the method as phenotypic confirmatory test for ESBLs is 87% to100% and the specificity is 95-100%. The availability of cefotaxime strips, as well as ceftazidime strips, improves the ability to detect ESBL types, which preferentially hydrolyzed cefotaxime, such as CTX-M type enzymes (Rawat and Nair, 2010).

2.9.4 .Vitek ESBL test

A specific card which includes tests for ESBL production has now been by approved FDA.The Vitek ESBL test (bioMerieux vitek, Hazelton, Missouri) utilizes cefotaxime and ceftazidime alone (at 0.5µg/ml) and in combination with clavulinic acid (4µg/ml). Inoculation of the cards is identical to that performed for regular vitek cards. Analysis of all wells is performed automatically once the growth control well has reached a set threshold (4-15 hours of incubation). A predetermined reduction in the growth of the cefotaxime or ceftazidime wells containing clavulanic acid, compared with the level of growth in the well the cephalosporin alone, indicates presence of ESBL. Sensitivity and specificity of the method exceed 90% (Rawat and Nair, 2010)

2.10. ESBLs prevention and control

Proper infection-control practices and barriers are essential to prevent spreading and outbreak of ESBL producing bacteria. The reservoir for these bacteria seems to be the gastrointestinal tract of patients. The contaminated hands and stethoscopes of healthcare providers are important factors in spreading infection between patients. Essential infection control practices should include avoiding unnecessary use of invasive devices such as indwelling urinary catheters, hand washing by hospital personnel, increased barrier precaution, and isolation of patients colonized or infected with ESBL producers. At an institutional level, practices that can minimize the spread of such organisms include clinical and bacteriological surveillance of patients admitted to intensive care units and antibiotic cycling; as well as polices of restriction, especially on the empirical use of broad-spectrum antimicrobial agent such as the third and fourth generation cephalosporin and quinolones (Rawat and Nair, 2010).

2.11. Previous studies

A Study done in Roosendaal in 2012, in total of 174 patients were included. In 24 of 174 (14%) patients, ESBL carriage could not be confirmed with the micro array. This was verified days, with PCR and sequencing. The mean duration of isolation was 15 adding up to a total number of isolation days of 2571. False-positive days of results according to the micro array resulted in a total of 279 days for the DDCT. Using unnecessary isolation for the E-test and 151 E-test to detect the presence of ESBL results in a false-positive outcome in 14% of the cases. This results in unnecessary isolation of patients, which can be omitted by using a genotypic method (Wintermans *et al*., 2012).

Study was done in Taiwan (February 2011) by Hsueh-Hsialo to detect the genes encoding extended-spectrum β-lactamases (ESBLs) and to determine the epidemiological relatedness of 69 *Escherichia coli* and 33 *Klebsiella pneumoniae* isolates collected from a regional hospital in central Taiwan, mostly from inpatients (*E. coli* 87.0%; *K. pneumoniae* 88.0%). The phenotypes of these isolates were examined according to the combination disc method recommended by the Clinical and Laboratory Standards Institute. Most of the ESBL-producing *E. coli* and *K. pneumoniae* isolates (98.6% and 97%, respectively) could be detected using cefotaxime discs with and without clavulanate. Genotyping was performed by PCR with type-specific primers. CTX-M-14 type (53.6%) was the most prevalent ESBL among *E. coli* isolates while SHV type (57.6%) was the most dominant among *K. pneumoniae* isolates. Six *E. coli* and three *K. pneumoniae* isolates did not carry genes encoding ESBLs of types TEM, SHV, CTX-M-3, CTX-M-14, CMY-2 and DHA-1. The co-existence of two or more kinds of ESBL in a single isolate was common, occurring in 40.6% and 72.7% of *E. coli* and *K. pneumoniae* isolates, respectively.

Study done in West India (2013) by Dr Akpaka in Female patients (67.8%) and urine samples (65%) yielded most ESBL isolates, with over 90% recovered from the hospital‘s medicine and surgery facilities. All ESBL isolates including all *K.pneumoniae* producing ESBLs were 100% susceptible to carbapenems and amikacin antimicrobials. Polymerase Chain Reaction detected 100% bla TEM genes, 4.1% *bla* SHV and 37.5% *bla* CTXM genes among *E. coli* isolates. Similarly, 84.3% *bla* TEM, 34.5% *bla* SHV and 58.8% *bla* CTXM genes were detected in *K. pneumoniae*.

Study done in West India from December 2004 to April 2008 found 602 *K .pneumoniae* and 1016 *E. coli* recovered from the clinical specimens were identified as ESBL producers. A 15.2% ESBL rate among the *K. pneumoniae* isolates and 9.3% among the *E. coli* isolates has previously been reported in this Hospital (Akpaka *et al.*, 2008)

Study done in North Eastern Italy reported that 70 ESBL-producing *E. coli*, 61 (87.1%) were positive for bla CTX-M, alone (30, corresponding to 42.8%) or in combination with bla TEM (31, 44.3%). Only 8 isolates showed other resistance genes (1 *bla* TEM alone, 1 *bla* SHV and 7 with associated *bla* TEM and *bla* SHV). In *K. pneumoniae*, *bla* SHV was present in all 5 isolates, alone or associated with *bla* TEM) or *bla* TEM and CTX-M. In *K. oxytoca*, 3 strains carried *bla* SHV and 1 *bla* TEM, while *bla* CTX-M was absent; only one strain resulted negative for all the ESBL tested (Busetti *et al.*, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design

This study is hospital and laboratory-based study.

3.1.1. Type of study

The study is descriptive, cross-sectional study.

3.1.2. Study area

This work was carried out in Military Hospital in Omderman, during the period from April to May 2013.

3.1.3. Study population

Pregnant women with urinary tract infection (UTI) were included in this study.

3.1.4. Sample size

One hundred mid –stream urine samples were collected from pregnant women

3.1.5. Data collection

Data were collected using interviewing questionnaire (Appendix 1)

3.1.6. Inclusion criteria

Pregnant women with UTI symptoms

3.1.7. Exclusion criteria

All pregnant women without UTI symptoms and pregnant women under antibiotic treatment.

3.1.8. Ethical considerations

Approval of the National Ethics Committee, Ministry of Health (Sudan).

3.2. Collection of specimens

5 ml midstream urine samples were collected in wide mouth screw-capped and leak-proof sterile containers containing 0.1g/10 ml boric acid as preservative.

3.3. Cultivation of specimens

The specimens were inoculated under aseptic conditions on CLED agar using sterile standard bacteriological wire loop. The inoculated culture media were incubated aerobically at 37Cº overnight and observed for bacterial growth.

3.4. Identification

3.4.1. Colonial morphology

Used as first identification depending on size, color, edges, side views and fermentation of lactose.

3.4.2. Microscopical examinations

Fixed and dried smear from the growth were prepared. Gram stain was performed for each slide as follows: slides were covered with crystal violet stain for 30-60 sec, then washed with water and covered with logol‘ s iodine for 30-60 sec, washed, decolorized rapidly (few second) with alcohol , washed immediately and covered with safranine for 2 minutes then washed and examined microscopically using oil immersion lens (X100). *E. coli* ATCC®25922 was used as control.

3.4.3. Biochemical identification

Biochemical tests were carried out according to Cheesbrough (2000) eg indole test, urease test, citrate, motility and the tube of KIA medium will show (slope and butt) for Gram negative. Catalase test, coagulase, DNase test and mannitol salt agar for Gram-positive.

3.5. ESBL screening test

All isolates were tested for their susceptibility to the 3rd generation cephalosporins, i.e.: ceftazidime (30µg), cefotaxime (30µg) and ceftriaxone (30µg) by the standard disc diffusion method as recommended by the CLSI guidelines (2010). ESBL were

screened by detection of reduced zones of inhibition around 3rd generation cephalosporins.

The bacterial isolate was considered as ESBL producer when the zone diameter for :ceftazidime < 22mm. cefotaxime < 27mm and ceftriaxone < 25mm. Resistant isolates to at least one of the 3rd generation cephalosporins were checked for ESBL production.

Double disk synergy test (DDST) was performed according to Jarlier *et al.*, (1988). Isolates were inoculated on Muller –Hinton agar plates. Discs containing ceftazidime (30Mg), cefotaxime (30Mg) and ceftriaxone (30mg) were placed 20 mm (center to center) away from a disc containing 20 mg amoxicillin/10mg clavulanic acid (AMC). Plates were incubated overnight at 37ºC. ESBL production was considered positive if the zone of inhibition around one or more of the 3rd antibiotic discs showed clear cut increase towards the AMCA disc (Ananthakrishanan *et al.*, 2000).

3.6. Genotypic characterization of ESBL genes

3.6.1. DNA extraction

1.5 ml of bacterial broth culture were transferred into eppendorff microfuge tubes and centrifuged at 12,000-16,000 for 2 min and then 200 µl of resuspension solution, 20 µl RNase (20 mg/ml), 25 µl lysozyme (50mg/ml), 20 µl proteinase K (20mg/ml) vortexed and incubated at 55ºC for 20 min.Then 200 µl lysis solution, was vortexed and incubated at70 Cº for 10 min. Prepared column by adding 500 µl of column preparation solution to the column, then centrifuged at maximum speed (13000 rpm). To bind DNA to the column 200 µl ethanol (100%) was added to the suspension, vortexed and transferred to the primed column, centrifuged again at(13000 rpm) for 2 min. The supernatant was discarded and 500 µl washing

solution were then added to the column , centrifuged at (9000 rpm) for 2 min, and then 500 µl washing solution were added to the column and centrifuge at 12000rpm for 3min ,discard and centrifuged at (13000 rpm) for 1min to dry the column. Was transfer the column to a new microfuge tube and 200 µl elution solution were added and centrifuged at 9000 rpm for 1 min.

3.6.2. Amplification of DNA using PCR

All isolates were screened for the resistance genes, *bla TEM*, *bla CTX-M* and *bla SHV* using PCR, according to Sidjabt *et al* .,(2009)(table1). The thermal cycler (CONVERGYS td peltier thermal cycle, Germany) was used throughout the study for the amplification of the PCR mixtures. In a total volume of 25µl containing 10 pmol of each three pairs of primers (Metabion, GERMANY), 4µl FIREPOL Master Mix (Solis BioDyne, Tartu, Estonia), 0.6 forward primer, 0.6reverse primer, 2µl plasmid DNA and 17.8 µl deionized sterile water. The PCR mixture was subjected to initial denaturation step at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 45sec, primer annealing at 55ºC for 45sec, followed by step of elongation at 72Cº for 60sec and the final elongation at 72ºC for 5min (Cao *et al*., 2002). PCR products were analyzed by electrophoresis in a 1% agaroses gel in TPE 1X, containing 0.5 mg/ml ethidium bromide at 120V for 40 min. Bands were visualized under U.V transilluminater (Uvite, UK).

Table1, Primers used for amplification of SHV, CTX-M and TEM genes

Gene Detected		Primer sequence (5___3)	Product size	Annealing temp	references
bla TEM	F R	5-TCG GGG AAA TGT GCG CG-3 5-TGG TTA ATC AGT GCA CC-3	971bp	55c	(Cao *et al* ., 2002)
bla SHV	F R	5-GGTTATGCGTTATATTCGCC-3 5-TTAGCGTTGCCAGTGCTC-3	850bp	55c	(Rasheed, *et al.*,2002)
bla CTX - M	F R	5-SCS ATG TGC AGY ACC AGT AA-3 5-CCG CRA TAT GRT TGG TGG TG-3	550bp	55c	(Cao *et al* ., 2002)

3.6 Statistical analysis

The data were recorded and analyzed. using SPSS statistical software, version 16(chi squer) And the results were presented in a form of tables and figures

CHAPTER FOUR

4. RESULTS

4. Results

Out of 100 urine specimens collected from the study population, bacterial growth was observed in 56 cultures. The results of Gram stain found 42(75%) Gram negative bacilli and14 (25%) Gram positive bacteria, the most of Gram negative was *K. oxytoca* 15(26.8%), *E.coli* 10(17.9%), *P. vulgaris* 8(14.3%), *K. pneumoniae* 5(8.9%), *Citrobacter frundii* 1(1.8%), *Enterobacter spp* 1(1.8%), *Providencea spp* 1(1.8%) and *Serratia marcesance* 1(1.8%). The most Gram positive *Staphelococcus saprophyticus* 13(23.2%) and *S. aureus* 1(1.8) (table2). The results of biochemical test for Gram negatives *E.coli, K, P.vulgaris, Citrobacter, Enterobacter , Provdencea and Serratia* (table3) and for Gram positive cocci *S.aureus* and *S.saprophyticus (*table4) The susceptibility test showed that 2(40%) of *K. pneumnae* was resistant to all antibiotic used (ceftazidime, cefotaxime and ceftriaxone) and 3(60%) *K. pneumoniae* was susceptible to all antibiotic used, all *K. oxytoca* was susceptible to all antibiotic used, *E.coli* 8(80%) were susceptible and 2(20%) were resistant, *P. vulgaris* 7(87.5%) were susceptible and 1(12.5%)was resistant, all *S. saprophyticus* were susceptabl and *Enterobacter, Providencea, Serratia, Citrobacter* and *S.aureus* were sensitive to 3rd generation cephalosporine used(table4). The ESBLs producer were screnning by resistant to 3rd generation cephalosporine found in 5isolates (11.9%) confirmed by using double disc synergy test (DDST) (figer1) found in 2 *K. pneumoniae,* 2 *E.coli* and 1 *P.vulgaris*. the PCR used to identifying genes resistant, The commonest prevalence of ESBL gene was TEM gene in all 5 organisms (figer2), CTX-M was produced by 3 organisms 2 *E. coli* and 1 *K. pneumoniae* and SHV gene in 1*K. pneumoniae* and 1 *P. vulgaris* (table5).

Table 2: The isolated organisms from this study

Isolates	Frequancy	Persent
Klebsiella oxytoca	15	26.8%
E. coli	10	17.9%
Proteus vulgaris	8	14.3%
Citrobacter frundii	1	1.8%
Enterobacter spp	1	1.8%
Providencea spp	1	1.8%
Serratia marcesance	1	1.8%
Klebsiella pneumniae	5	8.9%
S. aureus	1	1.8%
S.saprophiticus	13	23.2%
Total	56	100%

Table 3: Biochemical identification results of Gram negative bacteria

Isolates	Urease	Indole	Citrate	Motility	KIA			
					S	B	H2S	G
E .coli	_	+	_	+	Y	Y	_	+
Klebsiella pneumniae	+	_	+	_	Y	Y	_	+
Klebsiella oxytoca	+	+	+	_	Y	Y	_	+
Proteus vulgaris	+	+	_	+	R	Y	+	+
Citrobacter Freundii	+	_	+	+	R	Y	+	+
Enterobacter sp	_	+	+	+	Y	Y	_	+
Providencia sp	+	+	+	+	R	Y	_	+
Serratia marcescens	+	_	+	+	R	Y	_	+

S: slobe

B: bute

G: gase

Y: yellow

R: red

Table 4: Biochemical identification results of Gram-positive bacteria

Isolates	**Dnase**	**MSA**	**Coagulase**
S. aureus	+	+	+
S. saprophyticus	_	+	_

MSA: Manetol Solte Agar

Table 5: Antimicrobial susceptibility patterns of the isolated bacterial

Antibiotic / Bacteria	Ceftazidim		cefotaxime		Ceftriaxone	
	S	R	S	R	S	R
Klebsiella oxytoca	15(100%)	0	15(100%)	0	15(100%)	0
E.coli	8(79.9%)	2(19.1%)	8(79.9%)	2(19.1%)	8(79.9%)	2(19.1%)
Proteus vulgaris	7(87.5%)	1(12.5%)	7(87.5%)	1(12.5%)	7(87.5%)	1(12.5%)
Citrobacter frundii	1(100%)	0	1(100%)	0	1(100%)	0
Enterobacter spp	1(100%)	0	1(100%)	0	1(100%)	0
Providencia spp	1(100%)	0	1(100%)	0	1(100%)	0
Serratia marcescens	1(100%)	0	1(100%)	0	1(100%)	0

Klebsiella pneumoniae	3(60%)	2(40%)	3(60%)	2(40%)	3(60%)	2(40%)
S. aureus	1(100%)	0	1(100%)	0	1(100%	0
S.saprophyticus	13(100%	0	13(100%)	0	13(100%)	0

Table 6: TEM, SHV and CTX-M genes detected genotypically among ESBL producers

Gene type	*E.coli*	*K.pneumniae*	*P.vulgaris*
TEM	2(40%)	2(40%)	1(20%)
SHV	0	1(20%)	1(20%)
CTX-M	2(40%)	1(20)	0
TEM+CTX-M	2(40%)	1(20%)	0
TEM+SHV	0	1(20%)	1(20%)

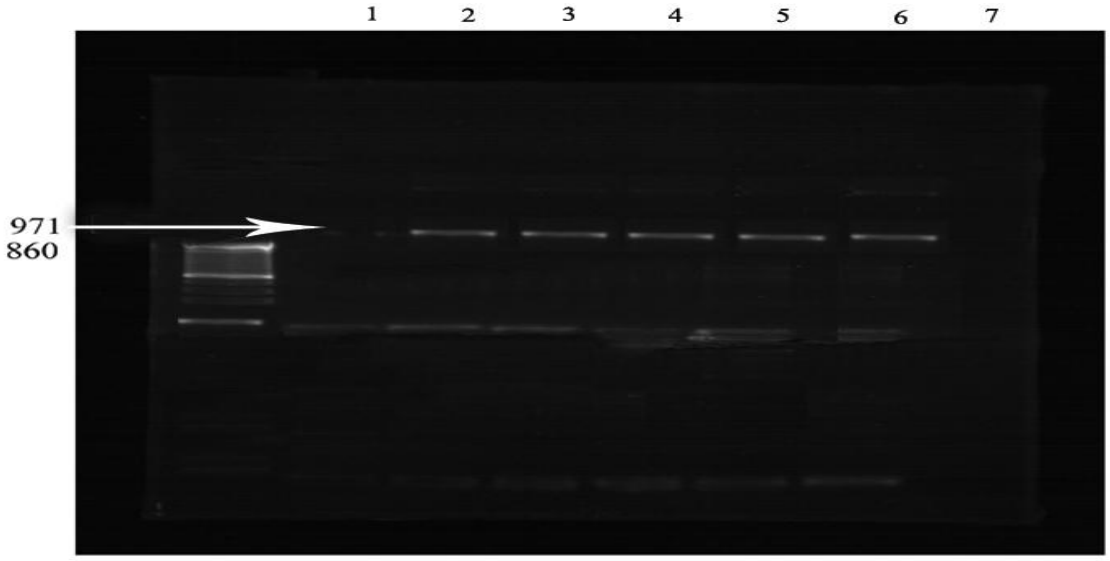

figure1: TEM gene after PCR on 1% agarose gel electrophoresis

Lane 1: negative control

Lanes 2,3,4,5 and 6: TEM gene positive

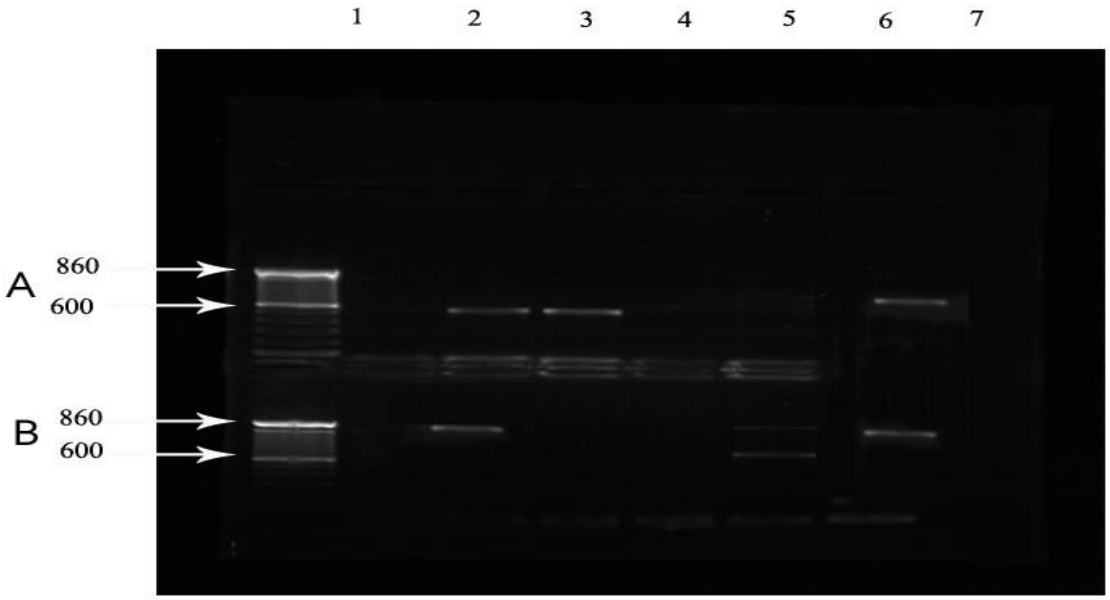

Figure 2: CTX-M and SHV genes after PCR on 1% agarose gel electrophoresis

Lane 1, A: negative control

Lanes 2, 3, and 6, A: CTX-M gene positive

Lanes 4and 5, A: CTX-M gene negative

Lane 1, B: negative control

Lanes 2 and 6, B: SHV gene negative

Lanes 3, 4 and 5, B: SHV gene negative

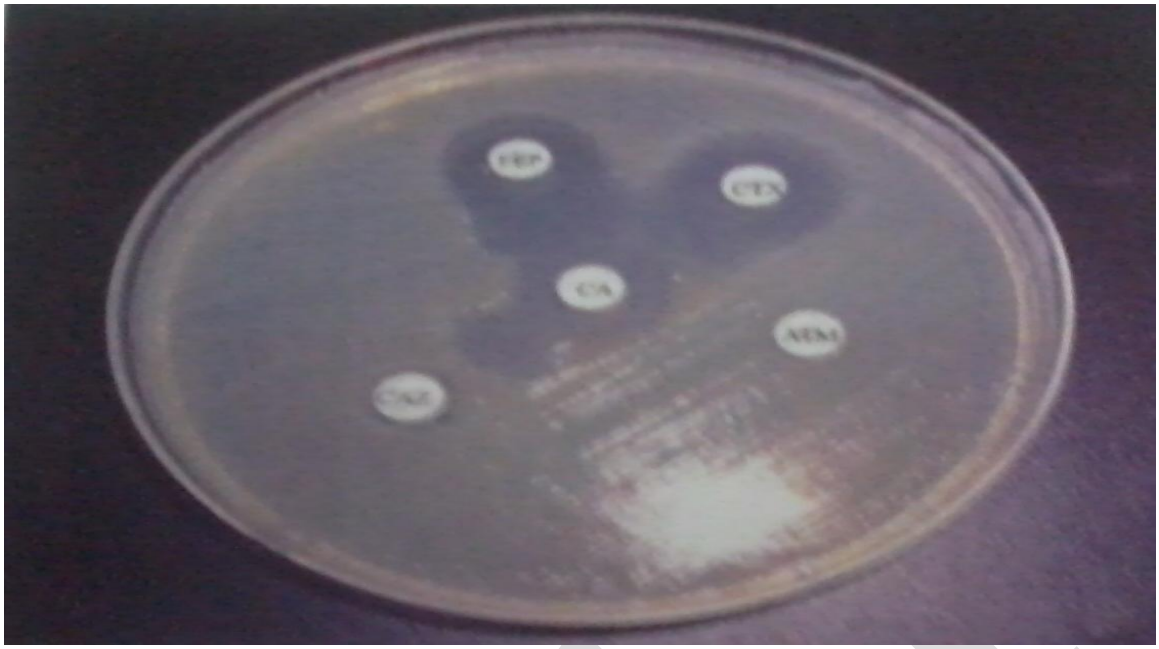

Figure 3: Double disc synergy test (DDS

CHAPTER FIVE

5.1. DISCUSSION

In this study out of 100 samples examined only 56 isolates identified, *K. oxytoca* 15(26%), *K. pneumoniae* 5(8.9%), *E. coli* 10(17.9%), *P. vulgaris* 8(14.3%), *Enterobacter spp* 1(1.8%), *Citrobacter freundii* 1(1.8%), *Provedancia spp* 1(1.8%), *S. aureus* 1(1.8%) and *S. saprophyticus* 13(23.2%). Many reports showed difference prevalence rates of ESBLs Enterobacterial uropathogens. In this study, 5(11.9) isolates producing ESBL were encountered, of which 2 (40%) *K. pneumoniae*, 2 (20%) *E. coli* and 1(12.5%) *P. vulgaris*, These results were similar to the combined disc method currently recommended by the NCCLS. For laboratories that perform susceptibility testing by using disc diffusion, modified DDST could be easily incorporated in to an already existing system. This finding was similar to study done by Taslima Yasmin (2012) in Bangladesh and Omer basher(2013) in Sudan reported that *E. coli* and *K. pneumoniae* are most ESBL producers.

Urine was the most main source of ESBL producing isolates, which is in agreement with that found by Akbar *et al* ., (2007), in Oman who reported that (70.4%) of urine was the main source of ESBLs from all patient.

The present showed that high prevalence of TEM gene. Which was Similar to that reported by Seker *et al*., (2009)? The CTX-M was the second ESBL gene detected in this investigation, a finding that was in agreement with that reported by Feizabadi, *et al.*, (2010). The present this study showed that CTX-M was most common gene among *E, coli* this was in agreement with that reported by Sajjad *et al* .,(2006)and Lavigne *et al* .,(2007). The SHV gene was the less frequent gene in this study, this result is similar with many reports around the world, such as Thailand by Kiratisine *et al* (2009) and in Iran (Dezful *et al.*, 2011). These results were not coniccding with those of Tasli and Bahar,(2005)and Ben-Ami *et*

al.,(2009), who detected SHV in 74.3% of isolates. This study is also disagreed with that reported in Sudan by Nour aldayem (2012) who found CTX-M is highly prevalent but agreed with SHV the less gene obtained.

5.2. Conclusions

In Sudan ESBL genes were among most strains of Enterobacteriaceae, especially *E. coli* and *K. pneumoniae.* ESBL SHV, TEM and CTX-M genes are predominant in Sudan among Enterobacteriaceae isolates and some strains carried one or more than of these genes and this may lead to the resistance to some cephalosporin antibiotic, that might complicate the management of the disease problem.

5.3. Recommendation

1- Formulation of proper antibiotic policy and providing appropriate guidelines to prescribe antibiotic, can prevent the spread of multi-drug resistant organisms in the hospital as well as in community.
2- Laboratory detection of ESBL producing bacteria is highly recommended.
3- DNA sequencing and specific target gene primers are required to differentiate between ESBLs and ESBLs variant of TEM and SHV genes.

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Appendix1

Questionnaire

Hospital……. date ……

Patientname……..…… NO…….

Age……..……. trimester ……...

Telephone…….

Residence …….

Diagnosis ……..

Clinical remark…….

……..

……..

Antibiotics administration ……

……

Type of antibiotic ……..

……

Time of administration……

Appendix 2

Media

A-CLED	100ML
B-Muller Hinton	
Beef dehydrated infusion	300g
Casein hydrolysate	1.5g
Starch	105g
Agar	10g
D.W	1L

c- Nutrient agar medium

peptic digest of animal tissue	5g/L
sodium chloride	5g/L
beef extract	1.5g/L
yeast extract	1.5g/L
agar	15g/L

Appendix-3

Reagent

A- normal saline

sodium	8.5
D.w	1L

B- alcohol 70%

Absolute alcohol	70ml
D.W	30ml

C-HCL

Concentrated HCL	8.6ml
D.W	100ml

D- kovac,s reagent

-4- dimethyle amino benzaldehyed	2g
isoamyle alcohol	30ml
concentrated hydrochloric	50ml

E- macfarland standard

Sulphuric acid solution	99.5ml
Barium chloride solution	0.5ml

Appendix 4

Gram stain

A-Crystal violet	20g
Ammonium oxlate	9g
D.W	1L
B - Loglos iodinen	
Potassium	2g
Iodine	10g
D.W	1L
C-safranine	
Safranine	0.5g
D.W	0.5ml