Sudan University of Science and Technology

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Molecular Detection of Extended- Spectrum Beta-Lactamases and *MCR-1* Genes in Gram negative Isolated from patient of Urinary Tract Infections in Khartoum State

الكشف الجزيئ على انزيمات البيتا لاكتام واسعة الطيف والجين المقاوم للكوليستين فى البكتريا سالبة الجرام والمعزولة من مرضى التهابات المسالك البولية فى ولاية الخرطوم

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بسم الله الرحمن الرحيم

قال تعالى:

آمَنَ الرَّسُولُ بِمَا أُنزِلَ إِلَيْهِ مِن رَّبِّهِ وَالْمُؤْمِنُونَ كُلٌّ آمَنَ بِاللَّـهِ وَمَلَائِكَتِهِ وَكُتُبِهِ وَرُسُلِهِ لَا نُفَرِّقُ بَيْنَ أَحَدٍ مِّن رُّسُلِهِ وَقَالُوا سَمِعْنَا وَأَطَعْنَا غُفْرَانَكَ رَبَّنَا وَإِلَيْكَ الْمَصِيرُ

صدق الله العظيم

سورة البقرة الآية 285

DEDICATION

The research work is dedicated to the beloved ones; my father, my mother, my husband, my daughter and my friends

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all thanks the Almighty Allah for making it possible for me to complete this work successfully. My heart full gratitude goes to Dr. HishamNouraldayem Altayeb for exellent supervision and for his unlimited support and guidance.

Abstract

This study aimed to detect extended specterum beta lactamase genes in Gram negative bacteria associated with Urinary Tract Infections in Khartoum State. Ninty one urine specimens were collected in this study. Samples were cultured on CLED agar and bacteria were identified by the conventional biochemical methods. Modified Kirby-Bauer method was usedfor sensitivity testing by using the following antibiotics: Gentamicin, Ciprofloxacin, Co-trimoxazole, Imipenem and Ceftazidime. A total of 44% of the isolates were sensitive to Gentamicin, 37% were sensitive to Ciprofloxacin, 45% were sensitive to Co-trimoxazole, 79% were sensitive to Imipenem and 27% were sensitive to Ceftazidime.

Boiling method was used for DNA extraction, and multiplex PCR was conducted to detect *TEM, AmpC, MCR-1, SHV* and *CTX-M* genes in Gram negative isolates. The majority of participants were females (49) while 42 males participated. Eighty nineisolates were positive to one or more ESBL gene while two samples were negative to all genes. The result of multiplex PCR came as follow: Fifty*E. coli*, 7 *Klebsiella pneumonia*, tow*Pseudomonas aeruginosa* and five*Proteus* species were positive to *CTX-M* gene. All *Citrobacter* isolates were negative to *CTX-M* gene. Forty five(49.4%) isolates were found positive for*AmpC* gene. Thirty one isolates (34.06%) were found positive for*TEM* gene,six (6.6%)were found positive for *SHV* gene, and thirty five (38.46%) were found positive for*MCR-1* gene. All *proteus* species were negative to *MCR-1* and *TEM* genes.As a conclusion, *CTX-M* gene is predominant among uropathogens and Imipenem has been found to be the best effective antibiotic.

المستخلص

هدفت هذه الدراسة لتحديد جينات البيتا لاكتاميز واسعة الطيف في البكتريا سالبة القرام المسببة لالتهابات المسالك البولية في ولاية الخرطوم . تم جمع 91عينة بول في هذه الدراسة.تم تزريع عينات البول على وسط CLED وتم التعرف على البكتريا بالطرق التقليدية . تم استخدام طريقة Kirby-buer المطورة لاجراء اختبار الحساسية باستخدام أقراص المضادات الحيوية التالية : جنتامايسين ' سبروفلوكساسين ' كوتراموكسازول ' اميبينيم و سفتازديم . ووجدنا ان 44% حساسة للجنتامايسين و37% حساسة للسبروفلوكساسين و45% حساسة للكوتراموكسازول و79% حساسة للاميبينيم و 27% للسفتازديم

تم استخدام الغليان لاستخراج الحمض النووي . أخيرا تم اجراء اختبار تفاعل البلمرة المتعددة المحتوى على عدة بادئات للكشف عن الجينات بيتالاكتميز واسعة الطيف (*SHVCTX-M* , *AMPC, MCR-1* , *TEM*) في الباكتيريا سالبة القرام

وكان معظم المشاركين في الدراسة من الاناث بعدد 49 وعدد الذكور 42 ، وكانت 89 عينة إيجابية لواحدة او اكثر من جينات بيتالاكتميز واسعة الطيف بينما كانت عينتان سلبية لجميع الجينات . نتيجة اختبار تفاعل البلمرة المتعددة المحتوى على عدة بادئات للكشف عن جينات بيتالاكتميز واسعة الطيف كالآتى : 50 من الاشريكية القولونية و7 من الكلبسيلة الرئوية .و 2 من الزائفة الزنجارية و5 من المتقلبة موجبة للجين *CTX-M* ولم تظهر نفس الجين في Citrobacter

45 عينة (49,4%) كانت موجبة لجين *AmpC* ,31 عينة (34,06%) كانت موجبة لجين TEM , ست عينات (6,6%) كانت موجبة لجين *SHV* بينما كانت 35 عينة (38,46%) موجبة لجين MCR-1. لم يتم العثور على جيني TEMوMCR-1 في باكتيريا المتقلبة الرائعة في الدراسة. وخلصت الدراسة الي ان الجين *CTX-M*منتشر بكثرة في البكتريا المسببة لالتهابات المسالك البولية وان المضاد الاميبينيم هو المضاد الحيوي الأكثر فعالية.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

Abbreviation	**Complete Words**
UTIs	Urinary tract infections
DNA	Deoxy ribose nucleic Acid
CTX-M	Cefotaximase.Munich
MCR-1	Mobilized Colistin Resistance
TEM	Temoneira
SHV	Sulfhydryl variable
UV	Ultra Violt

1. Introduction

1.1. Background

Urinary tract infection (UTI) is an infection caused by the presence and growth of microorganisms anywhere in the urinary tract. It is usually due to bacteria from the digestive tract which climb the opening of the urethra and begin to multiply to cause infection (Rahimkhani *et al*., 2008; Okonko *et al*., 2009). In contrast to men, women are more susceptible to UTI, and this is mainly due to short urethra, absence of prostatic secretion, pregnancy and easy contamination of the urinary tract with fecal flora (Haider *et al*., 2010) .(Urinary tract infection in pregnancy is associated with significant morbidity forboth mother and baby. The combination of mechanical, hormonal and physiologicchanges during pregnancy contributes to significant changes in the urinary tract, which has a profound impact on the acquisition and natural history of bacteriuria during pregnancy (Taher *et al*., 2009). Particularly in pregnancy, UTI may lead to unfavorable pregnancy outcomes and complications such as pyelonephritis, hypertensive disease of pregnancy, anaemia, chronic renal failure, premature delivery, low birth weight and fetal mortality (Delzell, 2000; Foxman, 2002; Smaill, 2007).

Increasing antibiotic resistance complicates its treatment by increasing patient morbidity, costs of reassessment and retreatment, rates of hospitalization, and use of broader-spectrum antibiotics (Hooton *et al*., 2004). Unfortunately despite the widespread availability of antibiotics, UTI remain the most common bacterial infection

among the human population. It is assumed that although being considered as benign diseases, in near future UTI will probably once become deadly disease and will be hardly treated. Culture and antimicrobial drug susceptibility testing are needed for surveillance purposes to guide the clinicians on the proper management and prevent empirical treatment to minimize further burden on antibiotic resistance (Colgan *et al*., 2006; Savas *et al.*, 2006).

ESBL producers have a wide clinical significance and high impact in healthcare systems especially in low income countries like poor access to drinking water, or a high population density are efficient driving forces for ESBL dissemination (Woerther *et al*., 2013). It is to be noted that ESBL producershave been reported to be associated with urinary tract infections among other different kind of infections such as pneumonia, , septicaemia, intra-abdominal infections and meningitis (Badal *et al*., 2013; Dayan *et al*., 2013).

1.2. Rationale

It is assumed that although being considered as benign diseases, in near future UTI, both the community acquired and in a large extent nosocomial UTI probably will once become deadly disease and will be hardly treated. The present study was designed to determine the bacterial profile and antibiotic susceptibility pattern of uropathogen among Sudanese of both sexes in several hospitals in Khartoum- Sudan, that will give an area based prevalence and antibiotic sensitivity pattern for empirical therapy.

1.3. Study Objectives

1.3.1General Objective

To detect ESBLs gene in Gram negative bacteria isolated from Urinary Tract Infected patients in Khartoum State.

1.3.2. Specific Objective

1. Isolation and identification of Gram negative bacteria in Urinary Tract infected patients.
2. To determine the susceptibility tests of the commonly used antibiotics against Gram negative bacteria.
3. To detect Extended Spectrum Beta Lactamase genes.(*AMPC,CTXM,TEM, SHV* and*MCR-1).*
4. To correlate between the presence of ESBL genes and isolated bacteria,gender,age and antimicrobial susceptibility.

2. Literature review

2.1. Definition and Etiological agents of UTI

Urinary tract infection is defined as the microbial invasion of any of the tissues of the urinary tract extending from the renal cortex to the urethral meatus. The urinary tract includes the organs that collect and store urine and release it from the body which include: kidneys, ureters, and bladder, urethra and accessory structures (Delzell, 2000). It is usually due to bacteria from the digestive tract which can ascend to the opening of the urethra and begin to multiply to cause infection (Rahimkhani *et al*., 2008).

Urinary tract infection can be either symptomatic or asymptomatic. Patients with significant bacteriuria and have at least two symptoms referable to the urinary tract infection (dysuria, urgency, frequency, incontinence, suprapubic pain, flank pain or costovertebral angle tenderness, fever (temp. $\geq$ 38^{o}C) and chills are said to be symptomatic. Asymptomatic bacteriuria (ABU) is a condition which is characterized by presence of bacteria in two consecutive clear-voided midstream urine specimens both yielding positive cultures ($\geq$10 cfu/ml) of the same uropathogen, in a patient without classical symptoms of UTI (Loh and Sivalingam, 2007).

Studies conducted in several parts of the world showed that *Escherichia coli* is the major etiologic agent in causing UTI, which accounts for up to 90% of cases (Gunther *et al*., 2001; Haryniewicz *et al*., 2001; Sahm *et al*., 2001; Getachew *et al*., 2012). *Proteus mirabilis, Klebsiella species, Pseudomonas aeruginosa* and *Enterobacter* species are less

frequent offenders of Gram negative bacteria. Less commonly, *Enterococci* and *Ureaplasma urealyticum* are also known causative agents in UTIs.

Gram-positive organisms are even less common in which Group B Streptococcus, *Staphylococcus aureus*, *Staphylococcus saprophyticus* and *Staphylococcus haemolyticus* are the recognized organisms (Loh and Sivalingam, 2007).

2.2. Epidemiology of UTI

It is estimated that 2 to 10% of pregnant woman suffer from any form of UTIs (Sheffield and Cunningham, 2005). These infections complicate up to 20% of pregnancies and are responsible for the majority of antepartum admissions to the maternal–fetal medicine units (Lee *et al.*, 2008). The prevalence of asymptomatic forms of UTIs has remained constant across countries, and most of the recent observational studies report similar rates, ranging from 2 to 10% similar to that of non-pregnant women (Wagenlehner *et al.*, 2009). Acute cystitis is prevalent in 1 to 4% of pregnant women (Duarte *et al.*, 2008).

Despite the relatively low prevalence of pyelonephritis (0.5 to 2%), it is estimated that 20% to 40% of women with asymptomatic bacteriuria will develop this condition later in gestation (Jolley and Wing, 2010). A study showed that if UTI is left untreated, 30% of mothers will develop acute pyelonephritis compared with 1.8% of non bacteriuric controls. Many studies have reported that pyelonephritis is more common during the second half of pregnancy, with an incidence peak during the last two trimesters of pregnancy (Gilstrap *et al.*, 1981). Acute pyelonephritis may lead to adverse outcomes for

the baby and the mother, such as premature delivery, low birth weight infants, preeclampsia, hypertension, renal failure and fetal death (Hill *et al.*, 2005).

2.3. Risk factors

2.3.1 Catheter

Bacteriuria develops in at least 10 to 15% of hospitalized patients with indwelling catheters. The risk of infection is ~3 to 5 % per day of catheterization. Many infecting strains display markedly greater antimicrobial resistance than organism that cause community acquired UTI. Factors associated with an increased risk of catheter associated UTI include female sex, prolonged catheterization, severe underlying illness, disconnection of the catheter and drainage tube, other types of faulty catheter care and lack of systemic antimicrobial therapy (Stamm, 2005).

2.3.2. Pregnancy

As in the general population, UTI is the most common infection in pregnancy (Andriole, 1991). Pyelonephritis during pregnancy requires hospitalization and is associated with complications such as premature labour and delivery. Even without pyelonephritis, studies suggest that there can be complications such as low birth weight, anemia, preterm labour and pregnancy-induced hypertension associated with ASB (Foxman, 2002).

2.3.3. Diabetes

Diabetics are more prone to infections in general and UTI is not an exception. Studies have shown that women who suffer from diabetes have ASB more frequently than

women without diabetes (Geerlings *et al*, 2000). Risk factors for UTI in diabetics include duration of diabetes, poor metabolic control of diabetes, sexual intercourse, and complications of diabetes. Fungal urinary infection is also more frequent in diabetics (Stapleton, 2002).

2.3.4. Obstruction

Any impediment to the free flow of urine like tumor, stricture, stone or prostatic hypertrophy- results in hydronephrosis and a greatly increased frequency of UTI. Infection superimposed on urinary tract obstruction may lead to rapid destruction of renal tissue.

2.3.5. Mechanical factors

Use of spermicidal compounds with a diaphragm or cervical cap or use of spermicide coated condoms dramatically alters the normal introital bacterial flora and has been associated with marked increases in vaginal colonization with *E. coli* and the risk of UTI.

2.3.6 Genetic factors

Research has shown that women with certain blood antigen (called lewis group) are more susceptible to cystitis. Cells that line their urinary tracts seem to have far more receptors to which bacteria can adhere. It has been demonstrated that nonsecretors of blood group antigens are at risk of recurrent UTI. Others may lack glycosamanoglycan, a substance found on the surface of the bladder that is inhospitable to bacteria. Mutations in host

genes integral to the immune response (interferon receptors and others) may also affect susceptibility to UTI (Stamm, 2005).

2.3.7. Menopause

Due to hormonal deficiency, changes in the genitourinary tract mucosa related to menopause may play a role in causing UTI.

2.3.8. Spinal cord injuries and multiple sclerosis

UTI is always complicated with spinal cord injury patients (Foxman, 2002). Many alterations in urinary tract function seen in spinal-cord injury patients, explain this high risk gives rise to problems such as incontinence, elevated intravesicular pressure, reflux, stones and neurological obstruction that increased susceptibility to UTI. Because of these abnormalities, most patients must resort to in this patient population is very important, since UTI are the second most frequent cause of mortality (Siroky, 2002). As with patients who suffer from spinal cord injuries, multiple sclerosis is also associated with an increased incidence of UTI (Foxman, 2002).

2.4. Pathogenesis of UTI

Urinary tract infections (UTIs) occur as a result of interactions between the uropathogen and host, and their pathogenesis involves several processes. Initially the uropathogen attaches to the epithelial surface; it subsequently colonizes and disseminates throughout the mucosa causing tissue damage. After the initial colonization period, pathogens can ascend into the urinary bladder resulting in symptomatic or asymptomatic bacteriuria.

Further progression may lead to pyelonephritis and renal impairment. Specific virulence factors residing on the uropathogen's membrane are responsible for bacterial resistance to the normally effective defense mechanisms of the host.

2.5. Clinical Presentations

Given that UTIs correspond to the growth and multiplication of bacteria within the urinary tract, it can be either symptomatic or asymptomatic. Asymptomatic bacteriuria (ABU) is a condition which is characterized by presence of bacteria in clear-voided midstream urine specimens which yielding positive cultures (≥10 cfu/ml) of the uropathogen, but without classical symptoms of UTI. Whereas symptomatic patients are characterized by presence of bacteria in clear-voided midstream urine specimens which yielding positive cultures (≥10 cfu/ml) of the uropathogen, and have at least two symptoms referable to the urinary tract infection (dysuria, urgency, frequency, incontinence, supra-pubic pain, flank pain or costovertebral angle tenderness, fever (temp ≥ 38^{o} C) and chills). (Anick *et al*., 2011). Then the resulting lesions can result in different degrees of severity. These infections can be grouped into different clinical entities, according to the anatomical location of injury (Anick *et al*., 2011).

Urinary Tract Infection is a broad term that encompasses different clinical sittings:

2.5.1. Urethritis

Urethritis is characterized by urethral colonization resulting in dysuria and polyuria. Approximately 50% of pregnant women suffering from this complication do not have

significant asymptomatic bacteriuria, and in 30% of them, urine cultures are negative. From a practical standpoint, only 20% of symptomatic patients have urine culture with more than 10 colonies/ml of urine. Another important detail is that some etiological agents involved in urethritis are normal floras commonly found in the vaginal cavity and that cause genital infections - some cannot be detected in routine urine cultures, such as Chlamydia trachomatis and Mycoplasma hominis. However, the potential invasiveness of these bacteria in the urinary tract is low (Anick *et al.*, 2008).

2.5.2. Cystitis

Cystitis is the infection of the bladder, occurring in about 1 to 1.5% of pregnancies. Common clinical manifestations are dysuria, polyuria, suprapubic discomfort, and in some cases hematuria (Le et al., 2004). Although dysuria and polyuria may suggest UTIs, these symptoms may concomitantly be present in pregnant women with other conditions, such as bacterial vaginosis (Nicolle, 2006). In addition, hemorrhagic cystitis during pregnancy can be confounded with bleeding issued from a process that could be bacterial, viral, fungal, immune (allergic) and radiotherapy. Cystitis is associated with preterm delivery and should be treated as soon as detected (Fakhoury *et al.*, 1994).

2.5.3. Pyelonephritis

Pyelonephritis is the most severe form of UTI in pregnant women and may affect up to 2% of this population. Its occurrence is directly associated with the prevalence of asymptomatic bacteriuria among pregnant women (Gilstrap *et al.*, 1981; Nowicki, 2002).

This condition can occur with or without symptoms of cystitis. Overall, pyelonephritis is associated with worse maternal and prenatal prognosis (Schieve *et al.*, 1994). Clinical signs and symptoms of pyelonephritis include flank pain (unilateral or bilateral) or abdominal pain, fever, anorexia, nausea and vomiting often associated with variable degrees of dehydration, chills, headache, and tachypneoa. Respiratory failure and sepsis can be present in severe forms. Fever is elevated in the acute forms (Rosen *et al.*, 2007).

2.6. Causative organisms

Most UTI is caused by a single pathogen, usually enteric gram-negative bacteria originating from the fecal flora of the host. The most common cause of uncomplicated UTI is *E. coli*, accounting for more than 80% of infections. *Staphylococcus saprophyticus* is the second most common pathogen, particularly among young, sexually active females, accounting for 5-15% of community-acquired episodes. This pathogen is generally considered more aggressive than *E. coli* because about 50% of the women present with upper tract involvement and infections due to this organism are more likely to be recurrent, relapsing, and persistent. Other bacteria such as *Klebsiella pneumoniae, Proteus mirabilis* and *Enterococcus faecalis* are occasionally involved.

Complicated UTIs including nosocomial infections are caused by more varied organisms and are generally more resistant than those causing uncomplicated infections. Although *E. coli* is still frequently isolated, it causes near about 50% of infections. Other organisms

that are frequently isolated include *Proteus* species, *Klebsiella pneumoniae, Enterobacter* species, *Pseudomonas aeruginosa, Staphylococci* and *Enterococci* (Bacheller, 1997; Barnett, 1997; Faro, 1998; and Mullenix, 1999).

Chlamydia trachomatis is an occasional causative agent found in women with dysuria, pyuria and negative cultures. For a diabetic patient, as an example, common infecting organisms include *E. coli*, as well as other organisms such as *Klebsiella, Enterococcus faecalis,* group B *Streptococci* and *Candida albicans* and these causal agents are found in greater frequency than in the general population. Yeast (usually *Candida albicans*), *Mycobacterium tuberculosis, Salmonella* species, *Leptospira* speciesor *Staphylococcus aureus* in the urine often indicate pylonephritis acquired via hematogenous spread. Less frequently isolated agents are other gram negative bacilli such as *Acinetobacter* and *Alkaligens* species, other *Pseudomonas* species, *Citrobacter* species, *Gardnerella vaginalis,* Beta hemolytic *Streptococci* and *Neisseria gonorrhoeae. Trichomonas vaginalis* may occasionally be observed in urinary sediment (Stapleton, 2002).

2.7. The most common bacteria causing UTI

2.7.1. *E. coli*

2.7.1.1. General properties

E. coli is widely distributed in the intestine of humans and warm blooded animals and is the predominant facultative anaerobe in the bowel and part of essential intestinal flora that maintains the physiology of the healthy host. Although most strains of *E. coli* are not

regarded as pathogens, they can be opportunistic pathogens that cause infections in immunocompromised hosts (Neill *et al*, 1994).

2.7.1.2. Pathogenesis of UTI by *E. coli*

Uropathogenic *E. coli* (UPEC) is responsible for approximately 90% of UTI seen in individuals with ordinary anatomy. In ascending infections, fecal bacteria colonize the urethra and spread up the urinary tract to the bladder. Uropathogenic *E. coli* (UPEC) utilize P fimbria (pyelonephritis-associated pili) to bind urinary tract endothelial cells and colonize the bladder. These adhesions specifically bind D-galactose- D-galactose-moieties on the P blood group antigen of erythrocytes and uroepithelial cells (Todar, 2007)

2.7.1.3. Antibiotic resistance

E. coli is resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotic resistance is a growing problem. Antibiotic resistant *E. coli* may also pass on the genes responsible for antibiotic resistance to other species of bacteria, such as *Staphylococcus aureus. E. coli* often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species of bacteria (Salyers*et al*., 2004).

2.7.2. *Klebsiella* species

Klebsiella species is second only to *E. coli* as a urinary tract pathogen. *Klebsiella* infections are encountered far more often now than in the past. This is probably due to the bacterium's antibiotic resistance properties. *Klebsiellae* species is known as a resident

of the intestinal tract in about 40% of man and animals. It is considered to be an opportunistic human pathogen (Eickhoff, 1972).

2.7.2.1. Resistance

Klebsiella bacteria are generally resistant to many antibiotics, such as penicillin. Often, two or more powerful antibiotics are used to help eliminate a *Klebsiella* infection (Brochert, 1999). *Klebsiella* possesses a chromosomal class A beta-lactamase giving it inherent resistance to ampicillin. Many strains have acquired an extended-spectrum beta-lactamase with additional resistance to carbenicillin, ampicillin, quinolones and increasingly to ceftazidime.

2.7.3. *Pseudomonasaeruginosa*

Pseudomonal infections of the urinary tract usually are hospital acquired and iatrogenic, related to catheterization, instrumentation and surgery. These infections can involve the urinary tract through an ascending infection or through bacteremic spread and are a frequent source of bacteremia (Qarah, 2005)

2.7.3.1. Resistance

Multidrurg resistant *Pseudomonas aeruginosa* is a highly relevant opportunistic pathogen which inherits efflux pumps with chromosomally-encoded antibiotic resistance property. One of the most worrisome characteristics of *P. aeruginosa* consists in its low antibiotic susceptibility. This low susceptibility is attributable to concerted actiongenes and the low permeability of the bacterial cellular envelops. Besides intrinsic resistance *P. aeruginosa*

develop acquired resistance either by mutation in chromosomally-encoded genes or by the horizontal gene transfer of antibiotic resistance determinants (Cornelis, 2008).

2.7.4. *Proteus* species

2.7.4.1 Pathogenesis of UTI by *Proteus*

UTI is the most common clinical manifestation of *Proteus* infections. The attachment of *Proteus* species to uroepithelial cells initiates several events in the mucosal endothelial cells, including secretion of interleukin 6 and interleukin 8. *Proteus* organisms also induce apoptosis and epithelial cell desquamation Urease production, together with the presence of bacterial motility and fimbriae, may favour the production of upper urinary tract infections (Gonzalez, 2006).

2.7.4.2. Resistance

Resistance to ampicillin and first-generation cephalosporins has been acquired by 10 to 50% of strains. Overall, 5% of *P. mirabilis* isolates in the United States now possess an ESBL. Resistance to ampicillin and first-generation cephalosporins is the rule for these species. Derepression of an inducible chromosomal *AmpC* ß-lactamase (not present in *P. mirabilis*) occurs in up to 30% of strains (Russo, 2005).

2.7.5. *StaphylococcusSaprophyticus*

Unlike other organisms commonly implicated in urinary tract infections, *S. saprophyticus* is not associated with hospital-acquired infections. Instead, colonization is community acquired, and infection occurs when the bacteria are introduced into the sterile urinary

tract. Patients with UTI caused by *S. saprophyticus* usually present with symptomatic cystitis. *S. saprophyticus* is usually susceptible to antibiotics commonly prescribed for patients with UTI, with the exception of nalidixic acid (Raz, 2005).

2.8. Beta-lactamases and their classification

The major defense mechanism that Gramnegative bacteria have against beta-lactam antibiotics is beta-lactamase production. The mechanism of the resistance is inactivation of the beta-lactam drugs by hydrolysis of the beta-lactam ring. The first beta-lactamase was identified in *E. coli*, before penicillin entered into clinical use in 1940 by Abraham and Chain (Abraham and Chain, 1940). Beta-lactamases can be classified according to two general schemes; the Ambler molecular classification scheme and the Bush-Jacoby-

Medeiros functional classification scheme (Ambler, 1980;Bush*et al.*, 1995).

According to the Ambler scheme, beta-lactamases are divided into four major classes (A to D). The basis of this classification scheme rests upon proteinhomology and not phenotypic characteristics. Class A, C and D are phylogenetically different serine beta-lactamases and class B is the class of metallo-beta-lactamases(Ambler, 1980). The Bush-Jacoby-Medeiros scheme classifies these enzymesaccording to functional similarity. This classification system is based on substrate profile and lactamase inhibitor susceptibility.

Beta-lactamases are plasmid-or chromosomally encoded enzymes; the genes encoding these enzymes were originally found on the bacterial chromosome (Bradford,2001). The first plasmid-encoded beta-lactamase, *TEM-1*, was described in early 1960s froma Greek patient named Temoniera, the enzyme was named after the patient. It was isolated from a single strain of *E. coli* and showed resistancemainly to aminopenicillins. Within a few years, the *TEM-1* beta-lactamase spread worldwideand is now found in different members of the *Enterobacteriaceae*family, as well as in *Haemophilus influenzae*, *Neisseria gonorrhoeae*and other Gramnegative pathogens(Liu*et al*., 1998).

Another enzyme group with the same resistance profile, *SHV-1*, was detected among*K. pneumoniae* and also in other members of the family *Enterobacteriaceae.*

Both genes coding for *TEM-1* and *SHV-* carried by conjugative transposons and plasmids, spread rapidly and became ubiquitous in Gramnegative bacteria, and *TEM-1*producing *K. pneumoniae* became endemic in many hospitals Over the last 20 years, many beta-lactam antibiotics have been developed to overcome the action of beta-lactamases. However, with each new class of antibiotics which has been used to treat patients, new beta-lactamases emerged to cause resistance(Heritage *et al*., 1999).

The selective pressure exerted by overuse of new antibiotics has been associated with the emergence of new variants of beta-lactamases. One of these new drug classes was (thirdgeneration) oxyimino-cephalosporins, which were widely used for the treatment of serious infections due to Gramnegative bacteria in 1980s (Liu*et al*., 1998).

In 1983, the first plasmid-encoded beta-lactamase capable of hydrolyzing the extendedspectrumoxyimino- cephalosporins was found in a strain of *K. ozaenae*in Germany, a mutatedform of the existing *SHV-1* enzyme (named *SHV-2*).

The first *TEM*-derived beta-lactamase conferring resistance to cefotaxime, *TEM-3* (initially named CTX-1), was reported in *K. pneumoniae* from France a few years later, and to distinguish these enzymes from broad-spectrum beta-lactamases the termextendedspectrum beta-lactamase was introduced by Philippon in 1989 (Philippon*et al*., 1989). In 1989, a non-*TEM*/*SHV*-producing*E. coli*isolate resistant to cefotaxime was recognized in Munich and was designated *CTX-M* due to its predominant activity against cefotaxime rather than ceftazidime (Bauernfeind*et al*., 1990).

2.8.1. *TEM*

TEM type ESBLs are derivatives of *TEM-1* and *TEM-2*. The first *TEM* beta-lactamase gene, designated as *blaTEM-1*, was isolated from a strain of*E. coli* in 1963 in Greece (Datta & Kontomichalou, 1965).

TEM-1, which is not an ESBL, can hydrolyze penicillins and first-generation cephalosporins; however, is unable to hydrolyze the oxyimino cephalosporins or monobactams (Sturenburg and Mack, 2003).

TEM-2 was the first derivative of *TEM-1*and had a single amino acid substitution at position 39; however ithad the same hydrolytic profile as *TEM-1*.

TEM-3 was the first *TEM*-type beta-lactamase that showed the ESBL phenotype.

Since that time the number and variety of extended-spectrum *TEM*-types has increased rapidly. More than 200 TEM type beta-lactamases have been described and new genes continue to appear (http://www.lahey.org/Studies/temtable.asp). These *TEM* variants differ in amino acid sequence, and many of them cause different resistance phenotypes. *TEM*-typeESBLsare most frequently found in *E. coli* and

K. pneumonia, but they are also reported in other Gram negative bacteria. *TEM*-type ESBLs have been identified in non-*Enterobacteriaceae* Gramnegative bacteria such as *Pseudomonas aeruginosa* (*TEM-42*) (Bradford, 2001).

2.8.2. *SHV*

The first emergence of an *SHV* ESBL was reported in Germany (Knothe*et al*., 1983), which was called *SHV-2*. This enzyme was found to differ from *SHV-1* (parent enzyme) byreplacement of glycin with serine at position 238 resulting in enhancement of the affinity of the*SHV-1* beta-lactamase to the oxyimino-cephalosporins. The majority of *SHV* variants showing an ESBL phenotype are identified by the substitution of a serine for glycine at position 238 and some of *SHV* variants have a substitution of lysine for glutamate at position 240. The serine residue at position 238 is critical for efficient hydrolysis of ceftazidime and lysine residue at position 240 is critical for efficient hydrolysis.Currently, more than 40 *SHV*-typeESBLshave been described

(http://www.lahey.org/Studies/) where changes in amino acid sequence confer the ability to hydrolyze the new cephalosporins (Sturenburg and Mack, 2003).

SHV-type ESBLs are mostly found in *K. pneumoniae*, but have also been found in *E. coli, Citrobacterdiversus* and *P. aeruginosa*.

2.8.3. *CTX-M*

The CTX-M type beta-lactamases was first recognized in 1989 as a new ESBL family member.The origin of *CTX-M*-type ESBLs was completely different from that of *TEM*- and*SHV*-type ESBL (Bonnet, 2004). The*CTX-M*family of enzymes is thought to have derived from initial transfer of the chromosomal beta-lactamase gene from *Kluyvera*species to conjugative plasmids that readily disseminated among different membersof the*Enterobacteriaceae* and other Gram negative bacteria. These original mobilized *blaCTX-M* genes affected cefotaxime to a higher degree thanceftazidime, and that is where the name came from, cefotaximase (Canton*et al.*, 2008).

From an evolutionary point of view, *CTX-Ms*, similarly to other ESBLs, later diverged by point mutations as a consequence of antibiotic selective pressure once *blaCTX-M* genes were mobilized from *Kluyvera* spp. and were incorporated into mobile genetic elements, which also gave them the opportunity to enhance the hydrolytic activity against ceftazidime like *CTX-M*-15, through acquisition of insertion sequences acting as strong promoters (Canton*et al.*, 2012).

To date there are over 160*CTX-M* variants (http://www.lahey.org/Studies/other.asp) recorded and they are divided into five different groups according to their amino acidsequences: i) *CTX-M*-1(emerged in Germany in 1989), ii) *CTX-M*-2 (emerged in Japan in 1986 and then in Argentina in 1989) , iii) *CTX-M*-8 (emerged in Brazil in 1996-1997), iv)*CTX-M*-9 (emerged in Spain in 1994) and v) *CTX-M*-25 (emerged in Canada in 2000) (Canton*et al.*, 2008). There has been a rapid spread of the *CTX-M* beta-lactamases, and they have been detected all over the world. The reason for this rise may be a) extraordinary dissemination of the corresponding *blaCTX-M*genes in highly mobilizable genetic platforms (plasmidsandtransposons), b) the presence of these platforms within successful clones (Canton and Coque, 2006; Rogers *et al.*, 2011), c) the co-resistance phenomenon in *CTX-M* producing isolates, especially to fluoroquinolones and aminoglycosides, which may facilitate a co-selection process (Canton *et al.*, 2012). The *CTX-M*-15 enzyme, which belongs to group 1, is considered to be the predominant ESBL type in most of the world, which has been referred as the *"CTX-M* pandemic" (Canton and Coque, 2006).

2.8.4. Other types of ESBL

Whereas the majority of ESBLs are coming from *TEM* or *SHV* beta-lactamases or are members of the *CTX-M* family, a few other ESBLs have been described that are not closely related to any of the above types of beta-lactamases. They are also plasmid-mediated, but are not derivatives of any other known betalactamase.

One of them is PER, which shares about 25% homology with the *TEM* and *SHV*-type ESBLs. PER- beta-lactamase was first detected in strains of *P. aeruginosa*isolated in Turkey. PER-2 which shares 86% homology to PER-1 has been detected more frequently in South America (Another type that is closely related to PER is VEB-1, which was first found in isolate of *E. coli* from Vietnam. Other VEB enzymes have also been detected in Kuwait and China (Bradford, 2001).

GES is a type of ESBLs that has recently been found in South America, Europe, South Africa and Japan. GES-1 beta-lactamase was first detected in a *K. pneumonia* isolate obtained in France in 1998. The blaGES-1 gene was subsequently detected in *P. aeruginosa* from France, and in *K. pneumoniae*from Portugal. Another variant, GES-2, was found in *P.aeruginosa* isolated in 2000 in South Africa, notably with the ability to confer intermediate resistance to imipenem (Poirel *et al*., 2001).

2.9. Clinical impact of ESBLs

ESBL producers have a wide clinical significance and high impact in healthcare systems especially in low income countries (as poor access to drinking water, poverty, and a high population density are efficient driving forces for ESBL dissemination). ESBL producers are associated with different kind of infections, such as pneumonia, urinary tract infections, septicemia, intra-abdominal infections and meningitis (Dayan*et al*., 2013).

ESBL producers often exhibit co-resistance to several commonly used antibiotic classes, such as fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole. This high resistance rate against drugs commonly used in the empirical treatment of critically ill patients may result in initial treatment failure, delay of adequate therapy and consequent increased morbidity and mortality rate,higher hospital costs and longer hospital stay. Thus, the treatment options are often very limited in infections caused by these bacteria.

Carbapenems have been regarded as the drug of choice for treatment of serious infections caused by ESBL-producing bacteria;however the disadvantage is the potential selection for carbapenem-resistance in ESBL producers and in other Gram negative bacteria (Cordery *et al*., 2008).

2.10. Laboratory Diagnosis

The laboratory investigation of microbial causes of UTI involves examining specimens to detect, isolate, and identify pathogens or their products using microscopy, culture techniques, and biochemical methods (Cheesbrough, 2006).

The diagnosis of UTIs begins with the screening of patients with symptoms suggestive of UTIs by a physician. Determination of the number and types of bacteria in the urine is an extremely important diagnostic procedure. Thus, only patients who have significant bacteriuria obtained from appropriate urine samples (a clean-catch midstream and catheter samples of urine) are included in the microbiological analysis. Bacteruria refers

to the presence of bacteria in the urine. It is regarded as significant when the urine contains 10 organisms or more per ml of pure isolates (Cheesbrough, 2006).

The type of media used for urine cultures is Cystine lactose electrolyte-deficient (CLED) agar. This media is now used by most laboratories to isolate urinary pathogens because it gives consistent results and allows the growth of both Gram-negative and Gram-positive pathogens. The indicator in CLED agar is bromothymol blue and therefore lactose fermenting colonies appear yellow. The medium is electrolyte-deficient to prevent the swarming phenomenon of *Proteus* species. All positive cultures are then identified at species level by their colony characteristics, gram-staining reaction and by the pattern of biochemical profiles using standard procedures (Cheesbrough, 2006).

2.11. Treatment and prevention of UTI

Use of low-dose antibiotics on a daily basis may be recommended to prevent UTIs if one gets frequent infections. Several nonspecific therapies have been used in preventing UTI and as an adjunct to antibiotics in treating UTI. Increased fluid intake is one such strategy, as this may result in removal of uropathogens by frequent voiding. Postcoital micturition may also flush bacteria from the bladder and urethra. Ascorbic acid may acidify the urine, thus increasing its antibacterial activity. More commonly, cranberry juice has been used to acidify the urine and has also been shown to prevent *E. coli* from adhering to the uroepithelium, thereby preventing infection. Additionally, urinary tract analgesics, such as Phenazopyridine, may be used to relieve dysuria. It has no antibacterial activity and is generally only used for one or two days. Patients should be

warned that Phenazopyridine may discolor the urine to a red-orange-brown color, which can stain clothing. Finally, estrogen therapy may prove useful in postmenopausal women with frequent UTI, as estrogens may decrease vaginal pH, thus increasing vaginal colonization with lactobacilli and suppressing vaginal growth of *Enterobacteriaceae.*

The development of resistance to amoxicillin is common among uropathogens and should be monitored. In the U.S., up to 33% of the uropathogens that cause UTIs are resistant to amoxicillin. Therefore, amoxicillin should be used only if susceptibility results are known. Tetracyclines and fluoroquinolones are contraindicated during pregnancy and should be avoided throughout all the developmental trimesters (Colgan et al., 2006). TMP-SMX can only be used safely during the second trimester. Use of TMP-SMX is discouraged during the first trimester due to possible teratogenic effects to the fetus, since trimethoprim is a folic acid antagonist (Hooton and Stamm, 2007). During the third trimester, use of TMP-SMX could displace bilirubin from its binding sites, resulting in kernicturus (bilirubin encephalopathy) (Colgan*et al*., 2006).

Since the presence of drug resistant bacteria in the environment is threat to the public, up-to-date information on local pathogens and drug sensitivity pattern is very crucial to manage patients. Bacteria become resistant to antimicrobial agents by a number of mechanisms, the commonest being: production of enzymes which inactivate or modify antibiotics, changes in the bacterial cell membrane, preventing the uptake of an antimicrobial, modification of the target so that it no longer interacts with the antimicrobial and development of metabolic pathways by bacteria (Cheesbrough, 2006).

Resistance in antimicrobial drugs in bacteria can result from two mutually nonexclusivephenomenons: mutations in housekeeping structural or regulatory genes and the horizontal acquisition of foreign genetic information. The rapid spread of antimicrobial resistance genes such as extended spectrum β-lactamases on mobile genetic elements such as plasmids and transposons is becoming a matter of concern for the probability of transmitting antimicrobial resistance from one microorganism to another worldwide (Courvalin and Trieu-Cuot, 2001).

To prevent UTI it is recommended that one should consider:

- Not to douche or use similar feminine hygiene products.
- Not to drink fluids that irritates the bladder, like alcohol and caffeine.
- Drink cranberry juice or use cranberry tablets, but not in case of personal or family history of kidney stones.
- Drink plenty of fluids.
- Keep the genital area clean.
- Urinate after sexual intercourse and
- Wipe from front to back can make one safe from UTI (Toedter, 2000).

3. Materials and Methods

3.1. Study design

Descriptive cross sectional study.

3.2. Study area

Study was conducted in Sherg elneel and Ribat hospitals. The centers are located in very active area in the center of Khartoum, with daily frequency of almost 400 patients visiting the centers for ophthalmologist, gynecologist, internist or urologist consulting, laboratory investigations including microbiology.

3.3. Study population

Participants of this study were patients with signs and symptoms of UTI and proceeded for culture inquiries. Samples of both males and females from all age groups were recruited.

3.4. Duration

For two successive months; from the 1st of April till the 30th of May, 2017, all patients visited the study area for urine culture inquiries were recruited to the study after their approval.

3.5. Data collection

3.5.1. Laboratory work

3.5.1.1. Specimen collection

For elimination surface contaminants before collecting urine specimens, only mid stream urine was collected in sterile containers for culture. An adequate amount of urine was taken by sterile dry Pasteur pipette for wet preparation.

3.5.1.2. Cultivation

The labeled specimens were immediately inoculated on CLED agar (Himedia laboratories Ltd, Mumbai 400086, India). Specimens were incubated aerobically for 24 hours at 37°C.

3.5.1.2.1. CLED agar

It is selecting, differential medium for growth and cultivation of urinary tract microorganism. The media is Electrolyte deficient for selection with the indicator bromothymol blue as to differentiate between lactose fermenting organisms and the non lactose fermenters.

3.5.1.3. Purification

Pure cultures of the pathogens were isolated by using nutrient agar plate. From the pathogenic bacteria vial by streaking on the nutrient agar medium pure isolated colonies

of pathogenic bacteria can be obtained. Further experiments such as antibiotic susceptibility test and biochemical tests were conducted using the pure culture growth.

3.5.1.4. Colonial morphology

The characteristics of growth on CLED agar were analyzed, i.e: color, 1.5mm diameter, opaque colony.

3.5.1.4. Microscopical examination

Gram stain which is essential technique for initial identification of bacterial isolates was introduced. The procedure was carried out as illustratedby (Cheesbrough, 2006) in the following procedure; smear was prepared from overnight culture on a clean and dry slide. The smear was left to air dry. Fixation was done by rapid pass of the slide three times through the flame of a Bunsen burner then allowed to cool before staining. Crystal violet stain was added to smear for 30–60 seconds, and then washed by tap water. Lugol's iodine was added for 30-60 minutes then washed by tap water and decolorized rapidly (few seconds) with acetone alcohol and washed immediately by tap water. Finally, the smear was covered with saffranin stain for two minutes and washed by tap water. The back of slide was wiped clean and placed in a draining rack for smear to air dry. Drop of oil was added to the dried smear and examined under the light microscope.

3.5.1.6. Biochemical tests

Using sterile straight twire loop The colonies were touched and inoculated on (kliger iron agar, tryptophan peptone water, semisolid media, Simmon's citrate agar, Christensen's urea agar) (HIMEDIA, India), and then incubated at 37 ºC over night incubation, then interpreted after adding Kovac's reagent to tryptophan peptone water medium .

3.5.1.7. *In- Vitro* antibiotic sensitivity testing

The standard disc diffusion method was used (Kirby-Bauer). The antibiotic discs used were from Himedia (Himedia Laboratories Pvt. Ltd, Mumbai 400086, India). The following antibiotics were used: Gentamicin (10mg), Ciprofloxacin (5mg), ceftazidime (30mg), imipenem (10mg) and co.trimoxazole (30mg).

The discs of the antibiotics were placed in the diagnostic susceptibility test agar (Muller Hinton Agar). The distance between the two adjacent discs was at least 20 mm .and from the edge of the plate was 15 mm. the media were incubated aerobically for 24 hours in 37°C .After 24 hours of incubation the diameter of the zone inhibition was measured and compared with the published tables of the control strains according to (CLSI guidelines, 2010).

Known control strain of *E. coli*ATCC 25922, was used for quality control.

3.5.1.7.1. McFarland standard

McFarland standards are extensively used as turbidity standards for the preparation ofsuspensions of microorganisms. The McFarland 0.5 standard has various applications in thepreparation of bacterial inocula for performing antimicrobial susceptibility testing. One of theearliest uses of turbidity for the enumeration of bacterial populations was in the preparation ofvaccines (Lorian, 1986). In 1907 McFarland developed a number of barium sulfate solutions toapproximate the numbers of bacteria in solutions of equal turbidity, as determined by platecounts (McFarland, 1907) (Forbes *et al*, 1998). The capability of susceptibility testing requiresthe use of standard inocula. The McFarland 0.5 standard was used for the preparation of inoculain the susceptibility tests for all isolated bacteria.

3.5.2. Molecular characterizations

3.5.2.1. DNA Extraction for Polymerase Chain Reaction

Genomic DNA templates for PCR amplification were gained from overnight growth of bacterial isolates on nutrient agar suspended in 1000 µL of sterile deionized water, and boiled for 15 minutes. After centrifugation of the boiled samples at 14000 g for 10 minutes, (Figure 10) supernatant was stored at -20°C as a template DNA stock(Yamamoto*et al.*,1995).

3.5.2.2. Gel electrophoresis of extracted DNA

The purity of the extracted DNA was determined by running the DNA sample on 1.5% gel agarose stained with ethidium bromide (20mg/dl), and then visualized under UV light (Sambrook*et al.*, 1989).

3.5.2.3. Preparation of 10 X TBE buffer

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

3.5.2.4. Preparation of 1X TBE buffer

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

3.5.2.5. Preparation of ethidium bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

3.5.2.6. Preparation of agarose gel

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 5 µl of (10mg\ml) Ethidium bromides were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles

were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed(Jalali et al.,2015).

Table 1: Amplicon sizes and primers used in the study

Primer	Sequence (5-3)	Amplicon Size Pb
TEM-F	ATGAGTATTCAACATTTCCGTG	861
TEM-R	TTACCAATGCTTAATCAGTGAG	861
SHV-F	ATTTGTCGCTTCTTTACTCGC	1050
SHV-R	TTTATGGCGTTACCTTTGACC	1050
AmpC-F	ATCAAAACTGGCAGCCG	550
AmpC-R	GAGCCCGTTTTATGGACCCA	550
CTX-M-F	SCSATGTGCAGYACCAGTAA	500
CTX-M-R	CCGCRATATGRTTGGTGGTG	500
MCR-1-F	CGGTCAGTCCGTTTGTTC	309
MCR-1-R	CTTGGTCGGTCTGTAGGG	309

3.5.2.7. Detection of resistance genes by Multiplex PCR

All subsequent multiplex PCR reactions were carried out using TECHNE® Ltd peltier thermal cycler (Germany), DNA amplifications was done using 5µl Maxime PCR PreMix kit (iNtRON, Korea). 1 µl DNA solution and 0.5 µl (10 pmol) of each gene-specific primer and 14µl D.W in a final volume of 25 µl. For ESBL genes detection, Primer-pair sequences (bla_{SHV}; bla_{TEM}; universal bla_{CTX-M}; bla_{MCR-1}; bla_{AmpC}) (Macrogen. Korea) were used in the multiplex PCR assay, primer sequences and expected PCR amplicon sizes are given in (Table 1). PCR amplification conditions were as follows: initial denaturation step at 95 °C for 5 minutes; 30 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute, extension at 72 °C for 1 minute, followed by a final extension step at 72 °C for 10 minutes as designed in the current study.

3.5.2.8. Agarose gel electrophoresis

In order to make 2% Agarose Gel; 1.0 gram of agarose was mixed with 50 ml 0.5x TBE buffer in an Erlenmeyer flask. Heat was applied for 2 minutes using microwave oven. Then it was Left to cool to 50^{o} C and 2.5 µl of 10 µg/ µl ethidium bromide solution was added. Mixed well and poured in gel pouring chamber. Two combs were then placed in the chamber and left to cool for about 30 minutes. Samples and DNA ladder (100-bp DNA ladder, iNtRON, Korea) were then loaded to the wells of the gel. Electrophoresis chamber was then Filled with the 0.5x TBE buffer.The gel electrophoresis apparatus

was connected to power supply (Primer, 100 V, 500 mA, UK). The electrophoresis was carried out at 75Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec – UK)(Jalali *et al*.,2015).

3.5.3. Statistical Analysis

Data were introduced to Statistical Package for Social Sciences (SPSS) software to estimate the *p* value of significance using Chi square test, means,frequencies and averages were also calculated.

3.5.4. Ethical Consideration

All patients (or co-patients) have been asked to sign consent prior to specimen collection. Verbal consent from laboratory administration has been also provided and approved the study.

4. Results

4.1. Demographic data

Total number of 91 urine samples has been collected from 42 males (46.3%) and 49 females (53.7%) and cultured during the study. 61 (67.03%) of samples were collected from Ribat hospital while 30 (32.97%) were collected from Sherg elneel hospital. The mean of the age distribution of the study population was (45.24) years old, (Figure 1).

4.2. The association between the presence of ESBLs genes and age group

The majority of participants of UTI patients infected with gram negative bacteria were in the 21-40 age interval46 participants representing 50.54%. Age group 1-20 contains only 8 participants (8.7%), 20 participants (21.97%) were found belonging to the age group 41-60 while the age group 61-80 has 16 participants (17.58%). The mean of the age distribution of the study population was 45 years old. The presence ofbla_{CTXM} gene has been found to be significantly associated with age (*P* value 0.03). All result is summarized in(Figure 1&Table 2).

Table 2: Result of the association between the presence of ESBLs genes and age group

Gene	Age group				P. value
	1 to 20	21 to 40	41 to 60	61 to 80	
AmpC	5	18	12	10	0.12
CTX-M	6	24	11	23	0.03
TEM	2	15	6	8	0.12
SHV	1	2	1	2	0.21
MCR-1	4	14	8	9	0.11

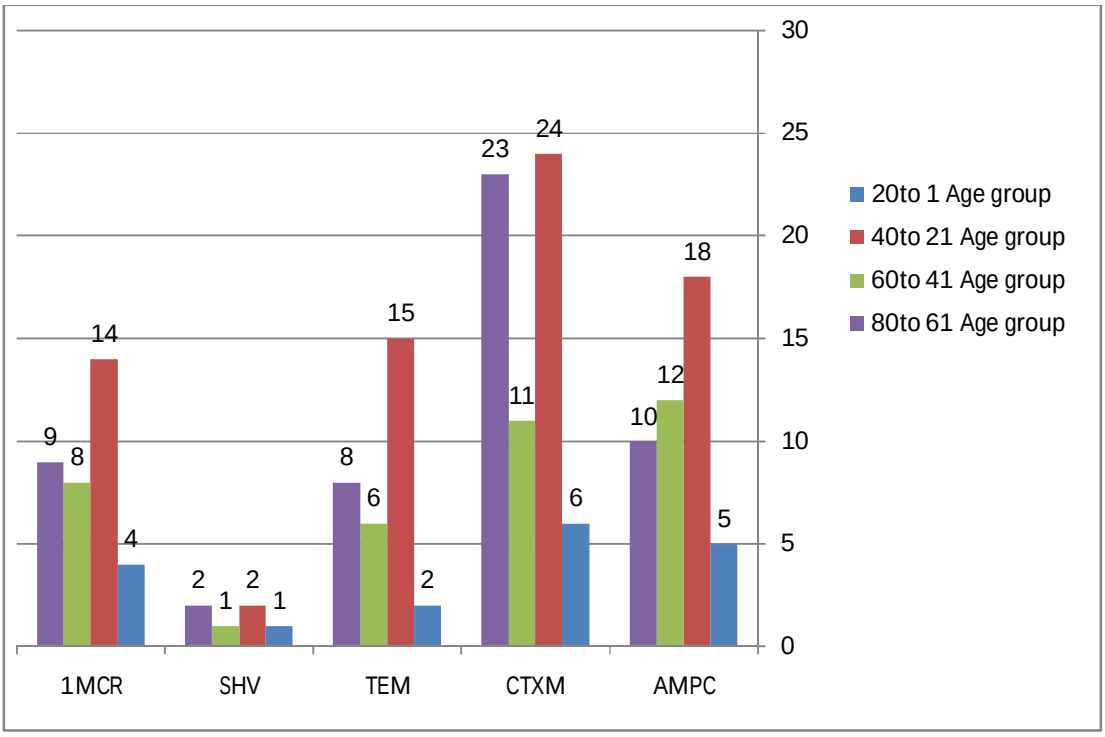

Figure 1: Gene's distribution according to age group.

4.3. The association between the presence of ESBLs genes and gender

The overall results revealed that total of 42 male, 49 female were positive for gram negative UTI. 26 (53.06%) females were positive for *AmpC* gene. 36 (73.46%) females were positive for *CTX-M* gene. 11 (22.44%) females were positive for *TEM* gene. 3 (6.12%) females were positive for *SHV* gene. 18 (36.73%) females were positive for *MCR-1* gene indicated in (Table 3& Figure 2). There was significant association between the presence of *TEM* and *CTX-M* genes and gender (*P* value= 0.02 and 0.04, respectively).

Table3: The association between the presence of ESBLs genes and gender

Gene	Male positive	Female positive	Male negative	Female negative	Total	P. value
AmpC	19	26	23	23	91	1
CTX-M	28	36	14	13	91	0.04
TEM	20	11	22	38	91	0.02
SHV	3	3	39	46	91	0.61
MCR-1	17	18	25	31	91	0.10

Figure 2: The association between presence of ESBLs genes and gender

4.4. Isolates

Ninety one samples revealed positive culture results under aerobic condition. All positive culture specimens showed single microbial growth. The commonest organism isolated was *E. coli* as 72 isolates (79.12%),*Citrobacter* one isolate,,*K.pneumoniae* 9 isolates, *P.auroginosa* 4 isolates ,*P.mirabilis* 2 isolates ,*P.vulgaris* 3 isolates (Figures 3 & Table 4).

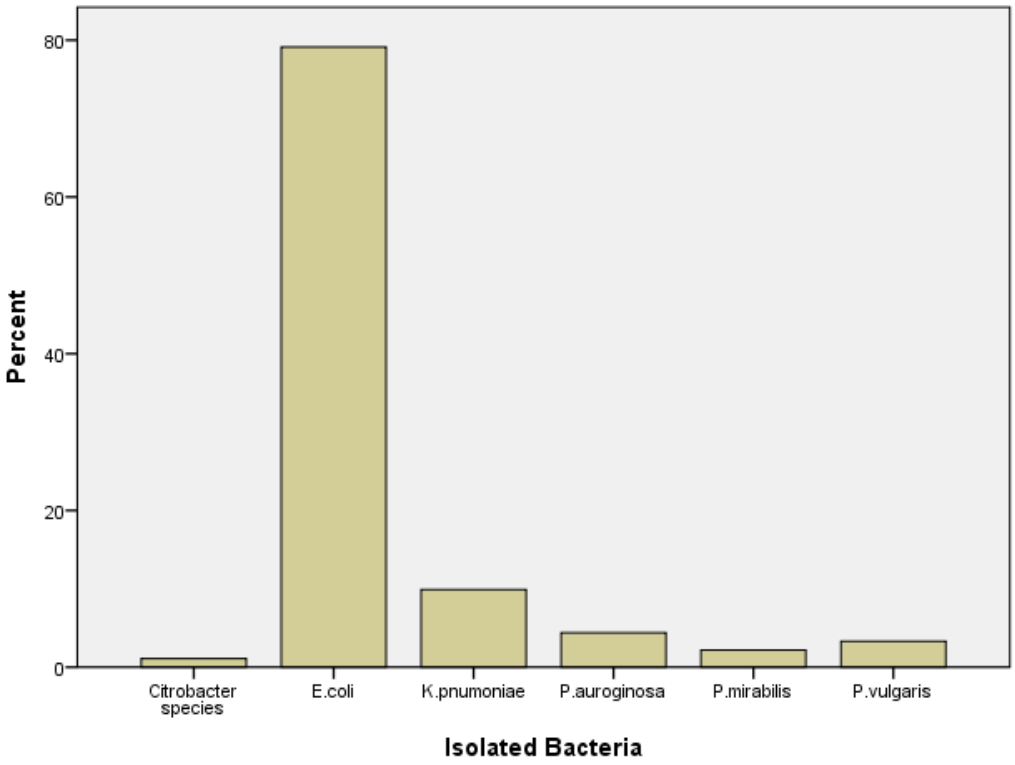

Figure 3: Percentage of different gram negative bacteria isolated in the study.

Among 72*E. coli* isolated; 50 isolates are showing positive result for the acquisition of bla_{CTX-M} gene (p= 0.03). Regarding *K.pnumoniae*; statistically significant relationship has been found between the organism and the presence of both bla_{CTX-M} and bla_{AmpC} (p= .03, .04, respectively). All results are summarized in (Table 4).

Table 4: Acquisition of ESBLs among study isolates.

Isolate		AmpC	CTX-M	TEM	SHV	MCR-1
Citrobacter species	**Positive**	0	0	1	0	1
	Negative	1	1	1	1	0
	P value	1.3	1.3	0.87	1.3	0.08
E.coli	**Positive**	32	50	24	5	31
	Negative	40	22	48	67	41
	P value	0.07	0.03	0.08	0.12	0.09
K.pneumoniae	**Positive**	6	7	2	0	1
	Negative	3	2	7	9	8
	P value	0.04	0.03	1.20	1.31	1.25
P.auroginosa	**Positive**	2	2	4	0	2
	Negative	2	2	0	4	2
	P value	1.2	1.2	0.00	1.24	1.2
P.mirabilis	**Positive**	2	2	0	0	0
	Negative	0	0	2	2	2
	P value	0.05	0.05	1.3	1.3	1.3
P.vulgaris	**Positive**	3	3	0	1	0
	Negative	0	0	3	2	3
	P value	.05	.05	1.3	1.2	1.3
Total	**Positive**	45	64	31	6	35
	Negative	46	27	60	85	56

4.5. Antimicrobial Susceptibility testing

Five antibiotic discswere used to perform antimicrobial susceptibility, Imipinem, Gentamicin, Ciprofloxacin , Co-trimoxazoleand Ceftazidime.

4.5.1. Imipinem

The overall results revealed that total of 79 samples are sensitive to Imipinem, among which 37 (46.83%) are positive for bla_{AMPC} gene, 55 (69.62%) are positive for bla_{CTXM} gene, 26 (32.91%) are positive for bla_{TEM} gene, 6 (7.59%) are positivebla_{SHV} gene, 31 (39.24%) are positive for bla_{MCR-1} gene, as indicated in Table 5& Figure 4).

Table 5: Association of presence of resistance genes and susceptibility toImipenem

Gene	Sensitive +ve	Sensitive -ve	Resistant +ve	Resistant -ve	Intermediate +ve	Intermediate -ve	P. value
AmpC	37	42	9	2	0	1	0.06
CTX-M	55	25	8	3	1	0	0.11
TEM	29	50	1	10	1	0	0.09
SHV	6	73	0	11	0	1	0.13
MCR-1	31	48	4	7	0	1	0.16

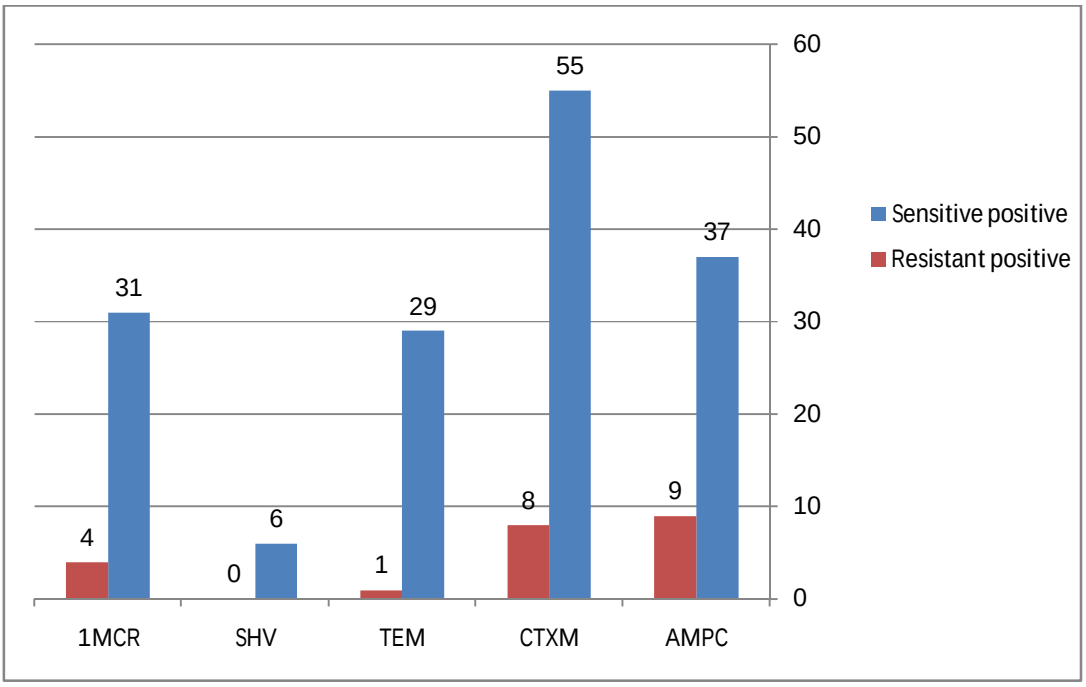

Figure 4: Result of Imipinem

4.5.2. Gentamicin

The overall results revealed that total of 44sensitive samples for Gentamicin, 21 (47.72%) are positive for bla_{AmpC} gene, 27 (61.36%)are positive for $bla_{CTX\text{-}M}$ gene, 11 (25%) are positive for bla_{TEM} gene, 0 (0%) are positive for bla_{SHV} gene and11 (25%) are positive for $bla_{MCR\text{-}1}$ gene. Statistically significant relationship between the presence of *MCR-1* gene and the resistance to Gentamicin is observed (P value = 0.03) as indicated in (Table 6&Figure5).

Table 6: Association of presence of resistance genes and susceptibility to Gentamicin

Gene	Sensitive positive	Sensitive Negative	Resistant Positive	Resistant negative	Intermediate positive	Intermediate negative	Total	P. value
AmpC	21	23	15	28	2	2	91	0.08
CTX-M	27	33	10	18	4	0	91	0.06
TEM	11	19	25	32	1	3	91	0.08
SHV	0	6	25	46	0	4	91	0.11
MCR-1	11	22	29	25	2	2	91	0.03

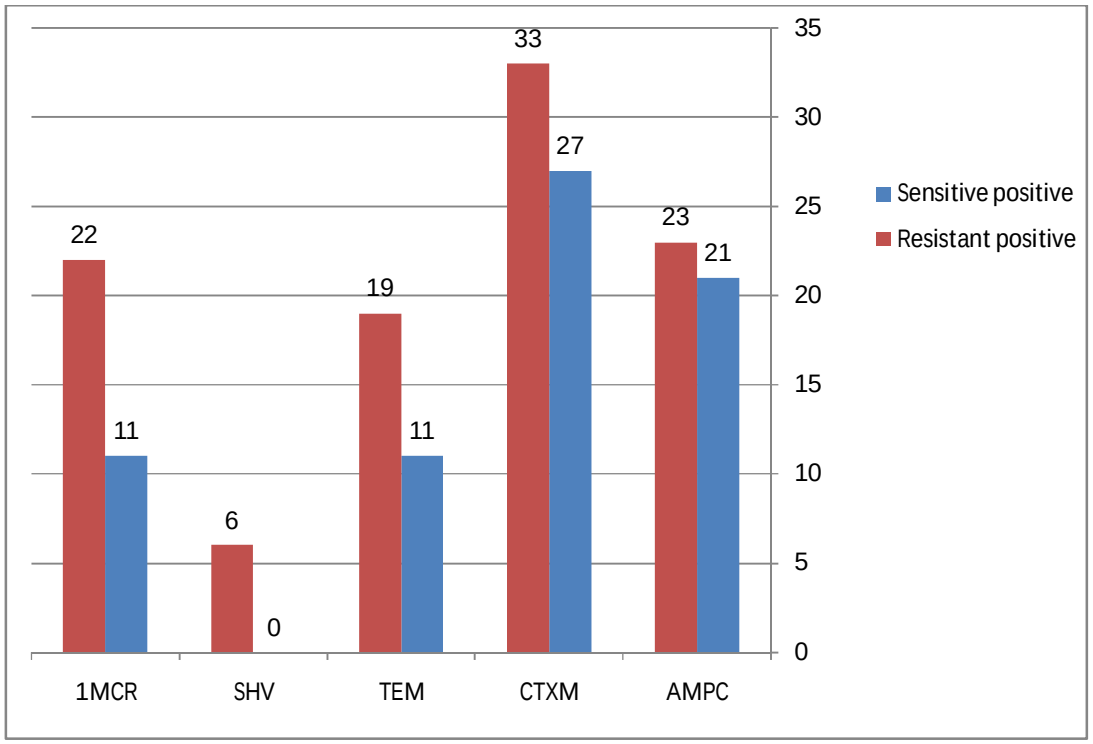

Figure 5: Result of Gentamicin.

4.5.3. Ciprofloxacin

The overall results revealed that a total of 37 sensitive samples for Ciprofloxacin, 18 (48.64%) are positive for bla_{AmpC} gene, 20 (54.05%)are positive for bla_{CTXM} gene, 8 (21.62%) are positive for bla_{TEM} gene, 0 (0%) are positive for bla_{SHV}, 8 (21.62%) are positive for bla_{MCR-1} gene. Statistically significant relationship between the presence of *CTX-M* gene and the resistance to Ciprofloxacin is observed (P value = 0.04)as indicated in (Table 7&Figure 6).

Table 7: Association of presence of resistance genes and susceptibility to Ciprofloxacin.

Gene	Sensitive positive	Resistant positive	Sensitive Negative	Resistant negative	Intermediate positive	Intermediate negative	Total	P. value
AmpC	18	25	19	26	2	1	91	0.09
CTX-M	20	42	17	9	2	1	91	0.04
TEM	8	23	29	28	0	3	91	0.09
SHV	0	6	37	45	0	3	91	0.14
MCR-1	8	26	29	25	1	2	91	0.08

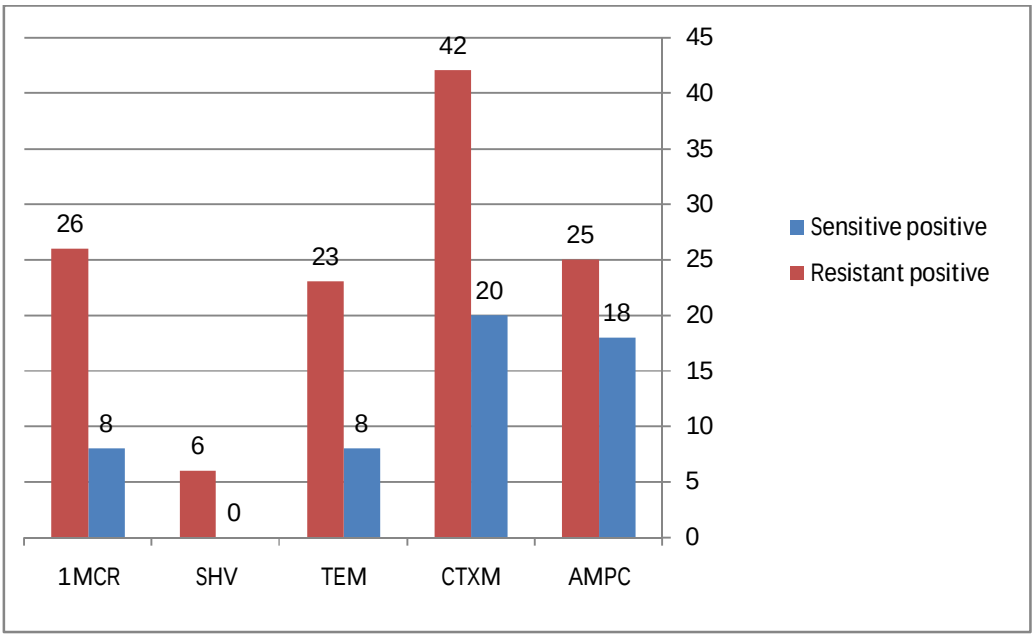

Figure 6: Result of Ciprofloxacin.

4.5.4. Co-trimoxazole

The overall results revealed that total of 34 sample sensitive for Co-trimoxazole, 15 (44.11%) are sensitive for bla_{AmpC} gene, 17 (50%) sensitive for bla_{CTXM} gene, 9 (26.47%) sensitive for bla_{TEM} gene, 1 (2.9%) sensitive for bla_{SHV} gene, 11(32.35%) sensitive for bla_{MCR-1} gene. Statistically significant relationship between the presence of *CTX-M* gene and the resistance to Co-trimoxazole is observed (P value = 0.05) as indicated in (Table 7& Figure 8).

Table 8: Association of presence of resistance genes and susceptibility toCo-trimoxazole.

Gene	Sensitive positive	Resistant positive	Sensitive Negative	Resistant negative	Intermediate positive	Intermediate negative	Total	P. value
AmpC	15	30	19	37	0	0	91	0.11
CTX-M	17	47	17	20	0	0	91	0.05
TEM	9	22	15	45	0	0	91	0.08
SHV	1	5	23	62	0	0	91	0.18
MCR-1	11	24	13	43	0	0	91	0.19

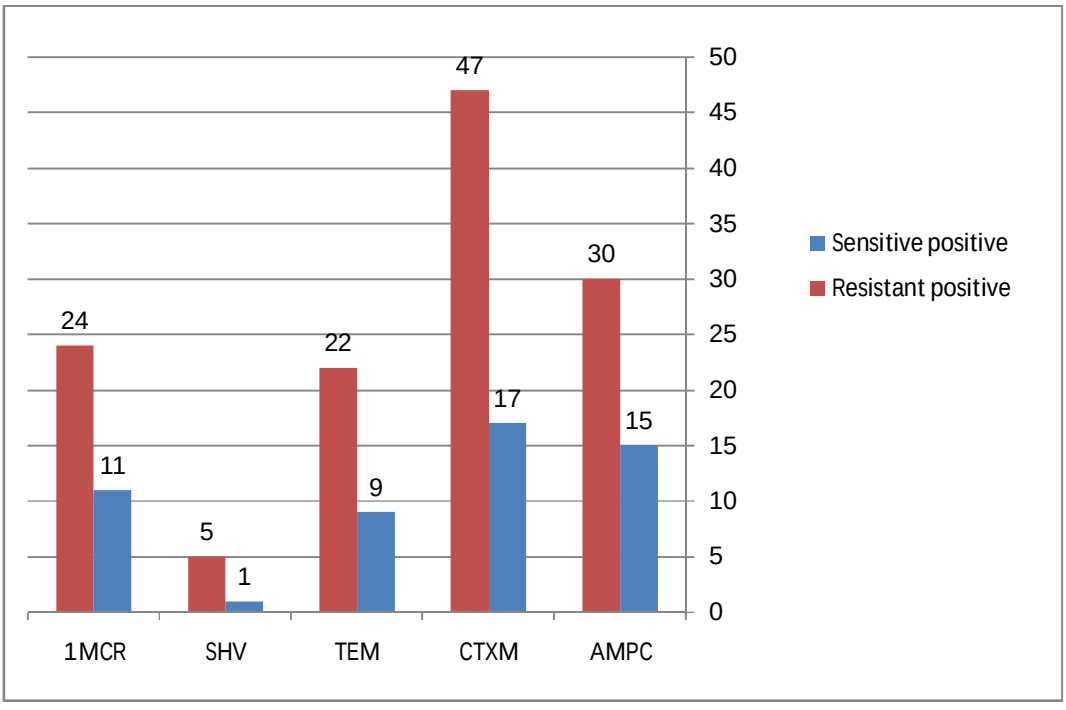

Figure 7: Result of Co-trimoxazole**.**

4.4.5. Ceftazidime

The overall results revealed that total of 27 sensitive samples for Ceftazidime, 11 (40.47%) are sensitive for bla_{AmpC} gene, 12 (44.44%) sensitive for bla_{CTX-M} gene, 6 (22.22%) sensitive for bla_{TEM} gene, 0 (0%) sensitive for bla_{SHV} gene, 8(29.62%) sensitive for bla_{MCR-1} gene. Statistically significant relationship between the presence of *CTX-M* gene and the resistance to ceftazidime is observed (P value = 0.03) as indicated in (Table 9& Figure 8).

Table 9: Association of presence of resistance genes and susceptibility to Ceftazidime.

Gene	Sensitive positive	Resistant positive	Sensitive Negative	Resistant negative	Intermediate positive	Intermediate negative	Total	P. value
AmpC	11	31	16	29	3	1	91	0.09
CTX-M	20	41	7	19	3	1	91	0.03
TEM	6	24	36	21	1	3	91	0.07
SHV	0	6	27	54	0	4	91	0.18
MCR-1	8	27	19	33	0	4	91	0.16

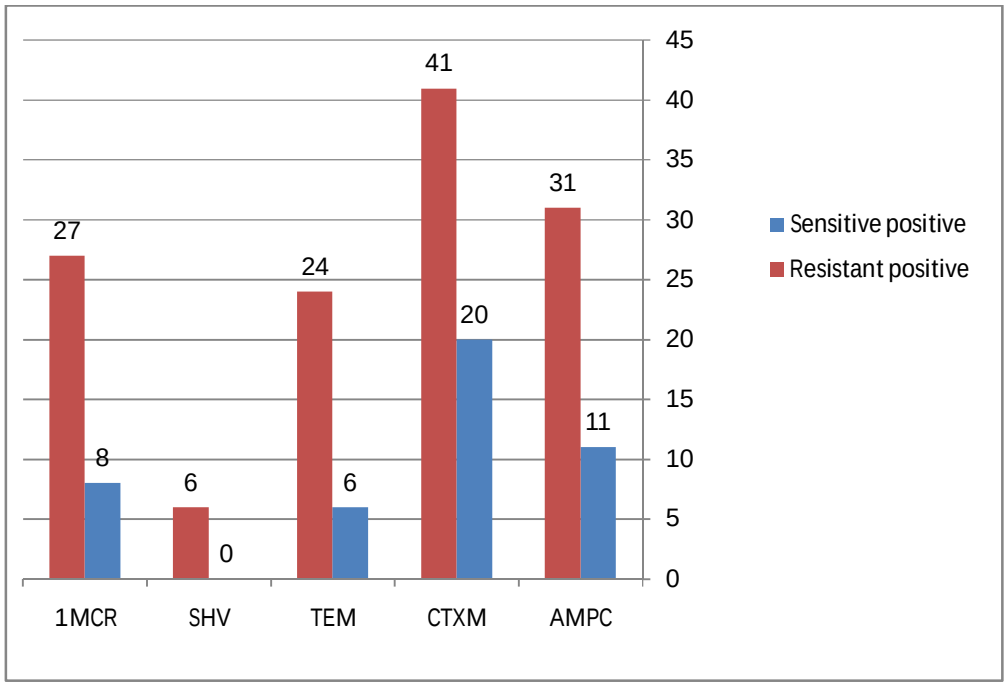

Figure 8: Result of Ceftazidime

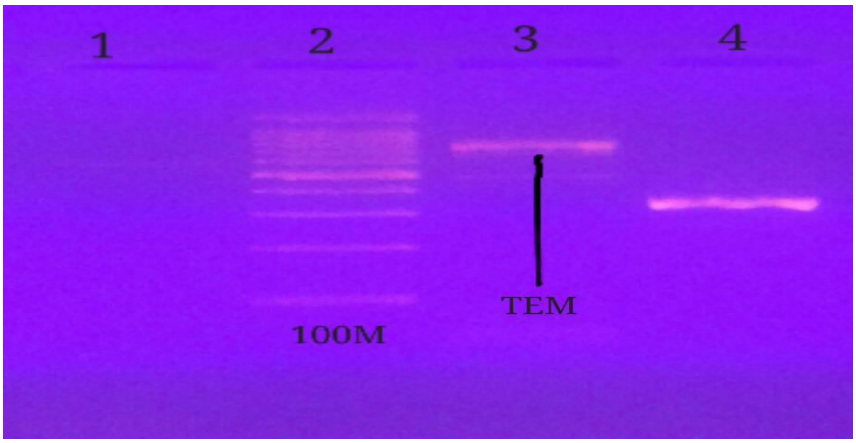

Figure 9: Agarose gel electrophoresis of multiplex PCR product of ESBL genes. 1= negative sample 2=100 bp ladder. 3= positive *TEM* gene. 4= positive*MCR-1*.

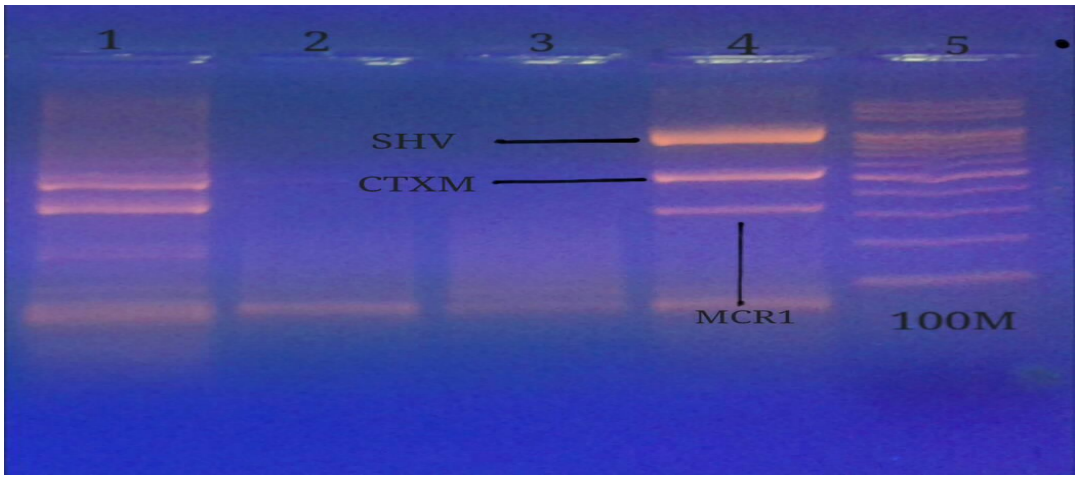

Figure11: Agarose gel electrophoresis of multiplex PCR product **1:***CTX-M*and *MCR-1*, ***2***and **3:** negativesample,**4:**positive*SHV* **,***CTX-M*and*MCR-1*,***5:***100bpladder**.**

5.Discussion.

For the frequency of ESBL genes, it is found that bla_{CTXM} is the most frequent among study isolates, as 64 isolates (70%) possess the gene. 45 isolates (50%) possess the bla_{AmpC} gene, 31samples (34%) possess the bla_{TEM} gene and 6 isolates (6.5%) possess the bla_{SHV} gene. However, antagonizing our findings, a recently published study in India concluded different frequencies of the tested ESBL genes as bla_{TEM}predominated as (48.7%), followed by bla_{CTXM} (7.6%) and bla_{SHV} (5.1%). However, their sample size was 38 samples (Bajpai*et al.*, 2017). In Sudan, (Ahmed *et al.*, 2013) demonstrated different frequencies as only (22%) were found positive for bla_{CTXM}, (17%) for bla_{TEM} and almost similar results for bla_{SHV} as (6.8%) of isolates were found positive. These differences might be due to the differences of the study time and population sample size as only 91 isolates are studied in the current study while 218 isolates were under the study of Ahmed and his colleagues.ESBL and AmpC production was measured in the study of (Yousif, 2015). Seventy eight (52%) strains produced ESBLs, whereas 4 (2.7%) organisms produced AmpC β-lactamase and six (4%) coproduced ESBLs and AmpC β-lactamases.This difference in AmpC gene prevalence might be due to the methodologies difference adopted in the current study and in Yousif and his colleagues' study; as they tested the isolates for AmpC beta-lactamasesusing the D68C *AmpC* andESBL detection set as well as testing the inducible *AmpC* beta-lacatmases using the disc antagonistic test. No study – to our knowledge has been found in literature describing the prevalence of *MCR-1* genes in Sudan.

In the present investigation, the most prevalent isolated bacterial uropathogen was *E .coli,* with an isolation rate of 30%, which is similar with previous studies (Hamdan *et al.,* 2011; Alemu *et al.*, 2012; Tazebew *et al.,* 2012). The major contributing factor for isolating at a higher rate of *E. coli* is due to a number of virulence factors specific for colonization and invasion of the urinary epithelium, such as the P-fimbria and S-fimbria adhesions (Sheffield and Cunningham, 2005).

Gender appeared to be a risk factor for the emergence of ESBL-producing bacteria, and the proportion of ESBLs in the current study was about 9% higher in females than in males, although some studies have shown no statistical difference in gender or a slightly higher incidence of ESBL-producing bacterial infection in men (Rodriguez-Bano *et al*., 2004; Calbo *et al*., 2006;Ortega*et al*., 2009; Azap*et al*., 2010).For instance, (Shah *et al*., 2002) studied the relation of ESBL-producing *Enterobacteriaceae* with respect to gender and reported more ESBL-positive isolates in males (65.33%) than females (34.67%). Das andBorthakur also found a slight male preponderance for ESBL production among the study subjects(Das & Borthakur, 2012). Our findings antagonize those studies as we observed female preponderance in the present study. After applying the*Z*-test for proportions with 95% CI, this difference was found to be statistically significant (P = 0.03). However, the low sample size in the current study not to be underestimated.

The current situation of resistance to antibiotics has reached a serious point in urinary tract infection, and presently, multidrug-resistant bacteria including ESBL-producing bacteria can be readily encountered in clinics. Antibiotics that can be used for the

treatment of multidrug-resistant bacteria including ESBL-producing bacteria in urinary tract infection are limited (Kader & Kumar, 2005).In our study, excluding imipenem, antibiotics with sensitivity higher than (60%) to ESBL-producing isolates were absent. Only 21 isolates (58%) from ESBL-producing organisms were sensitive to Gentamicin, (49.6%) to Ciprofloxacin, and 80% to Imipinem. Ceftazidime is effective only against 26.19% of ESBL-producing organisms in the study.Thus, low sensitivity of ESBL-producing *Enterobacteriaceae* has been observed for Gentamicin, Ciprofloxacin and Ceftazidime. Rudresh and Nagarathnamma reported a similar susceptibility patterns for ESBL isolates with 46.9% isolates sensitive to gentamicin followed by ciprofloxacin (29.5%) and Ceftazidime (23.4%). Moreover, Das and his team in their study have showed 30.96% sensitivity toward Ceftazidime (Rudresh & Nagarathnamma, 2011; Das & Borthakur, 2012).In Sudan, (Ibrahim *et al*., 2013)concluded that even 81% of ESBL producing isolates are resistant to Ciprofloxacin compared to 49.06% observed in the current study.Nevertheless, as Ibrahim and colleagues adopted the double-disk diffusion method to confirm ESBL production using antimicrobial disks of ceftazidime and cefotaxime, there might be a source of false positive results.

6.1. Conclusion

- There is high prevalence of ESBLs producer Gram negative isolates in Sudan.
- Among (72) *E. coli* isolated; 50 isolates are showing positive result for the acquisition of bla_{CTXM} gene (p= 0.03). Regarding *K.pnumoniae*; statistically significant relationship has been found between the organism and the presence of both bla_{CTXM} and bla_{AMPC} (p= .03, .04, respectively).
- There was significant association between the presence of *TEM* and *CTX-M* genes and gender (*P* value= 0.02 and 0.04, respectively).
- Statistically significant relationship between the presence of *MCR-1* gene and the resistance to Gentamicin is observed (P value = 0.03)**.**
- Statistically significant relationship between the presence of *CTX-M* gene and the resistance to Ciprofloxacin is observed (P value = 0.04).
- Statistically significant relationship between the presence of *CTX-M* gene and the resistance to Co-trimoxazole is observed (P value = 0.05).
- Statistically significant relationship between the presence of *CTX-M* gene and the resistance to ceftazidime is observed (P value = 0.03).

6.2. Recommendations

- If antibiotic treatment for UTIs is deemed necessary, then positive urine culture should be obtained beforehand, and the antimicrobial susceptibility patterns of uropathogens should be ascertained.
- Well oriented strategies to control the mass behavior regarding recurrent use of antibiotics are mostly needed.
- More comprehensive surveys should be carried out, in order to assess further risk factors in more details among Sudanese population.

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Appendix I – questionnaire

Sudan University of Science and Technology

College of Graduate studies

Molecular Characterization of Extended –Specterum Beta-Latamase of Gram Negative Isolates of Urinary Tract Infections in Khartoum State

By: Suhani Salah Mohamed Ali

Supervised by: Hisham Nouraldayem Altayeb Mohammed

Name…….. Date:……

Index number: ……

Age:……

Gender:……

Symptoms:

1-fever …….. 2- back pain…….

3-burning sensation …… 4- headache……..

Any treatment received……..

Previous diagnosis of UTI ……..

Culture result ……..

Sensitivity result……..

Signature:……..

Appendix II

Reagents and Stains

Gram Stain (Cheesebrough, 2000)

Most bacteria can be differentiated by their Gram reaction due to differences in the cell wall structure into Gram positive which after being stained dark purple with crystal violet are not decolorized by acetone or ethanol and Gram negative which after being stained with crystal violet lose their color when treated with acetone or ethanol and stain red with safranin.

Requirements

Crystal violet Gram stain (Hi Media)

To make 1 liter:

Crystal violet…… **... ... .** …20 g

Ammonium oxalate…… **.....**……9 g

Ethanol or methanol, absolute…… **.....**… **....**….95 g

Distilled water…… to 1 liter

lugol's iodine (Hi Media)

To make 1 liter:

Potassium iodide…… **.....**……20 g

Iodine……..10 g

Distilled water…….. to 10 liter

70% alcohol

Absolute alcohol…… **.....**…..70 ml

Distilled water…… **.....**……30 ml

Safranin (HiMedia)

Method of Preparation

• The dried smear was fixed by heat.

• The fixed smear was covered with crystal violet for 30-60 minutes.

• The stain was washed off with clean water.

• All water was tipped and the smear covered with lugol's iodine for 30-60 minutes.

• The stain was washed off with clean water.

• 70% alcohol was rapidly applied for 10-20 seconds for decolourization and then washed rapidly with clean water.

• The smear then covered with safranin stain for 2 minutes.

• The stain was washed off with clean water, back of slide was cleaned.

• After air-dry, smear was examined microscopically by using X 100 lens.

Results

Gram negative rods.

Preparation of Turbidity Standard

- 1% v/v solution of sulpharic acid was prepared by adding 1 ml of concentrated sulfuric acid to 99 ml of water. Mix well.
- 1.17% w/v solution of barium chloride was prepared by dissolving of 2.35g of dehydrated barium chloride (BaCl2.2H2O) in 200 ml of distilled water.
- To make the turbidity standard 0.5 ml of barium chloride solution was added to 99.4 ml of the sulpharic acid solution. Mix well.
- A small volume of the turbid solution was transferred to screw-caped bottle of the same types as used for preparing the test and control inoculate (Chemie, 2014).

Preparation of Media (Chemie , 2014)

CLED Agar (Cystine Lactose Electrolyte Deficient)

Formula in grams per liter (PH 7.4)

Lactose.....10,00

Gelatin Peptone 4,00

L-Cystine 0,128

Bacteriological Agar 15,00

Casein Peptone 4,00

Beef Extract..... 3,00

Bromothymol Blue 0,02

Preparation

Suspend 36 grams of the medium in one liter of distilled water. Soak 10-15 minutes and mix well. Heat slowly while stirring frequently boil for a minute. Sterilize in the autoclave at 121°C (15 lbs. of sp.) for 15 minutes. Pour into Petri dishes. When the medium is solidified, invert the plates to avoid excess moisture.

Kligler Iron Agar

Formula in grams per liter

Peptone mixture 20,00

Sodium Chloride..... 5,00

Ferric Ammonium Citrate 0,50

Phenol Red 0,025

Lactose 10,00

Dextrose 1,00

Bacteriological Agar 15,00

Sodium Thiosulfate.....0,50

Preparation

Suspend 52 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation. Boil for one minute. Dispense into tubes and sterilize at 121º C (15lbs. pressure) for 15 minutes. Allow to cool in a slanted position so as to obtain butts of 1’5-2 cm. Depth. For greater accuracy, Kligler Iron Agar should be used on the day of preparation or melted and solidified before use.

Tryptophan Culture Broth

Formula in grams per liter (PH 7.5)

Casein Peptone 10,00
L-Tryptophan 1,00
Sodium chloride.....5,00

Preparation

Suspend 16,0 grams of medium in one liter of distilled water. Heat to boiling agitating frequently. Distribute in test tubes, 3 ml each. Close the tubes with cotton or with a plastic or metallic cap. Sterilize at 121º C (15 lbs. sp.) for
15 minutes.

Simmons Citrate Agar

Formula in grams per liter (PH 7)

Ammonium Dihydrogen Phosphate 1,00
Dipotassium Phosphate..... 1,00
Sodium Chloride.....5,00
Sodium Citrate 2,00
Magnesium Sulfate.....0,20
Bacteriological Agar..... 15,00
Bromthymol Blue.....0,08

Preparation

Suspend 24,3 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation until completely dissolved. Dispense in tubes and sterilize in the autoclave at 121ºC (15 lbs sp.) for 15 minutes. Cool the tubes in a slanted position so that the base is short (1-1,5 cm. deep). Alternatively, the media can be poured into petri plates.

Christensen's Urea Agar

Formula in grams per liter (PH 6.9)

Gelatin Peptone..... 1,00
Dextrose 1,00
Sodium Chloride.....5,00
Monopotassium Phosphate 2,00
Urea 20,00
Phenol Red.....0,012

Preparation

Dissolve 29 grams of the medium in 100 ml. of distilled water. Sterilize by filtration. Separately dissolve 15 grams of agar in 900 ml. of distilled water by boiling. Sterilize in autoclave at 121°C (15 lbs.sp) for 15 minutes. Cool to
50°C and add to the 100 ml. of the sterile Urea Agar Base. Mix well and dispense aseptically in sterile tubes.
Leave the medium to set in a slanted position so as to obtain deep butts. At a pH of 6.8 to 7.0 the solidified medium should have a light pinkish yellow colour. Do not remelt the slanted agar.

Mueller-Hinton Agar

Formula in grams per liter (PH 7.4)

Beef, infusion …….300.0g

Cas amino acids……17.5 g

Starch……..1.5g

Agar …….17.0g

Distilled water……1000ml

Preparation

38.0 g of media was suspended in 100 ml distilled water. Sterilized by autoclaving at 15Ib pressure (121˚C) and poured in sterile petri dishes.

Appendix III

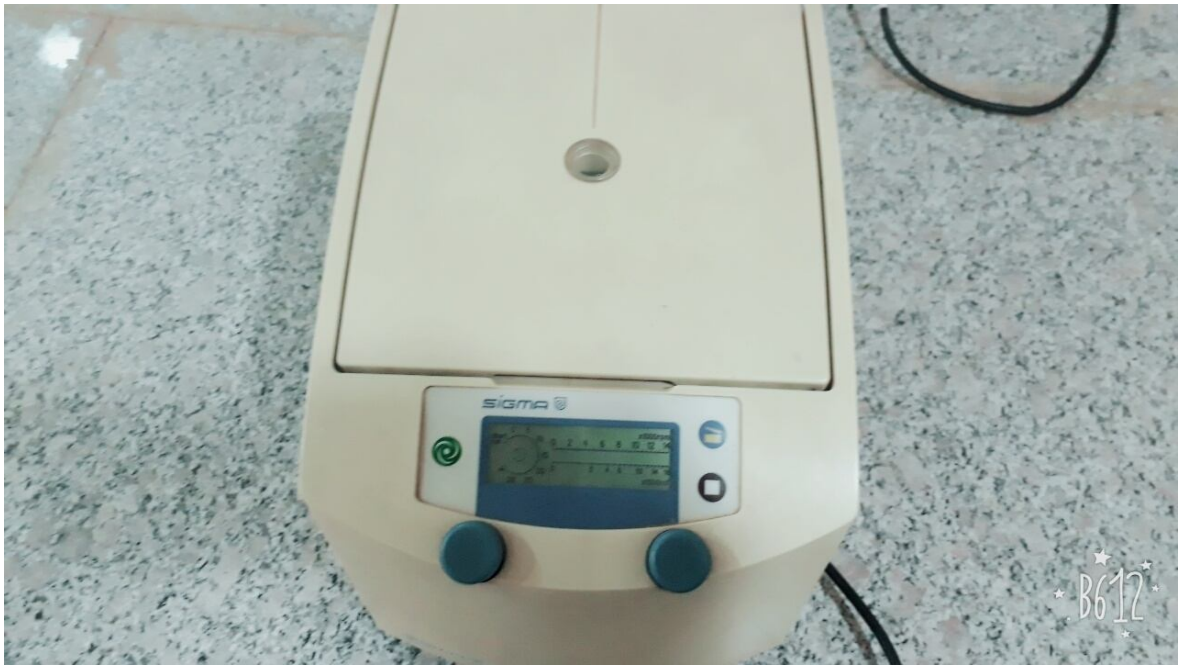

Figure (1) Microcenterfuge device

Figure (2) Thermocycle device

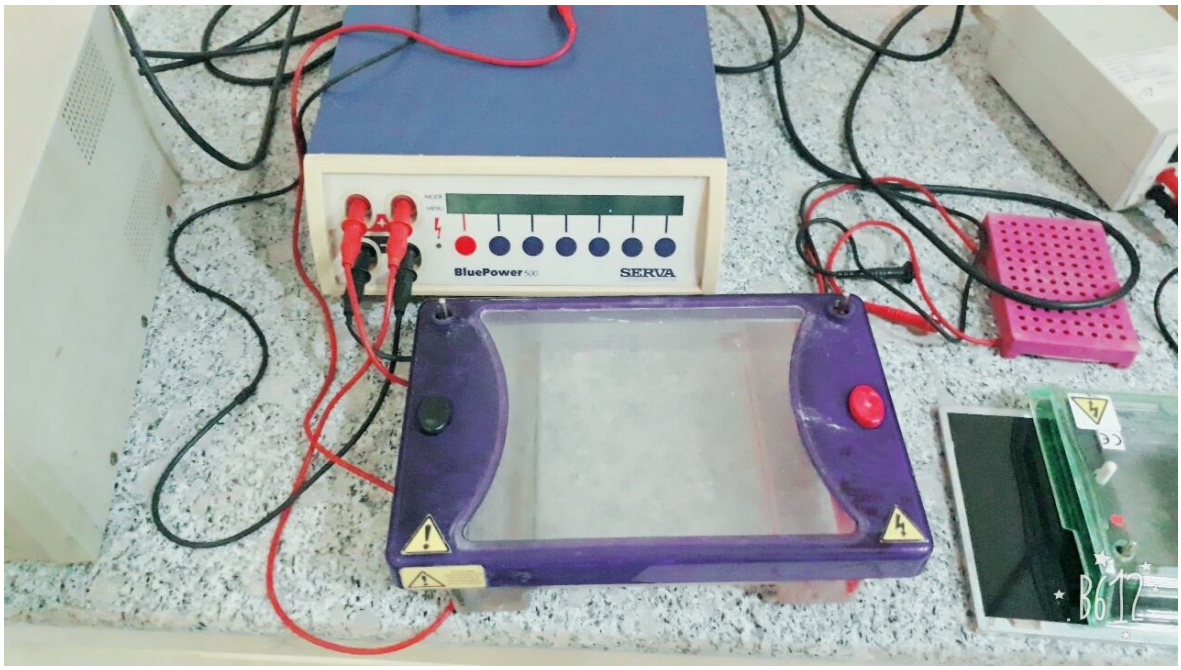

Figure (3) gel electrophoresis and power supply device

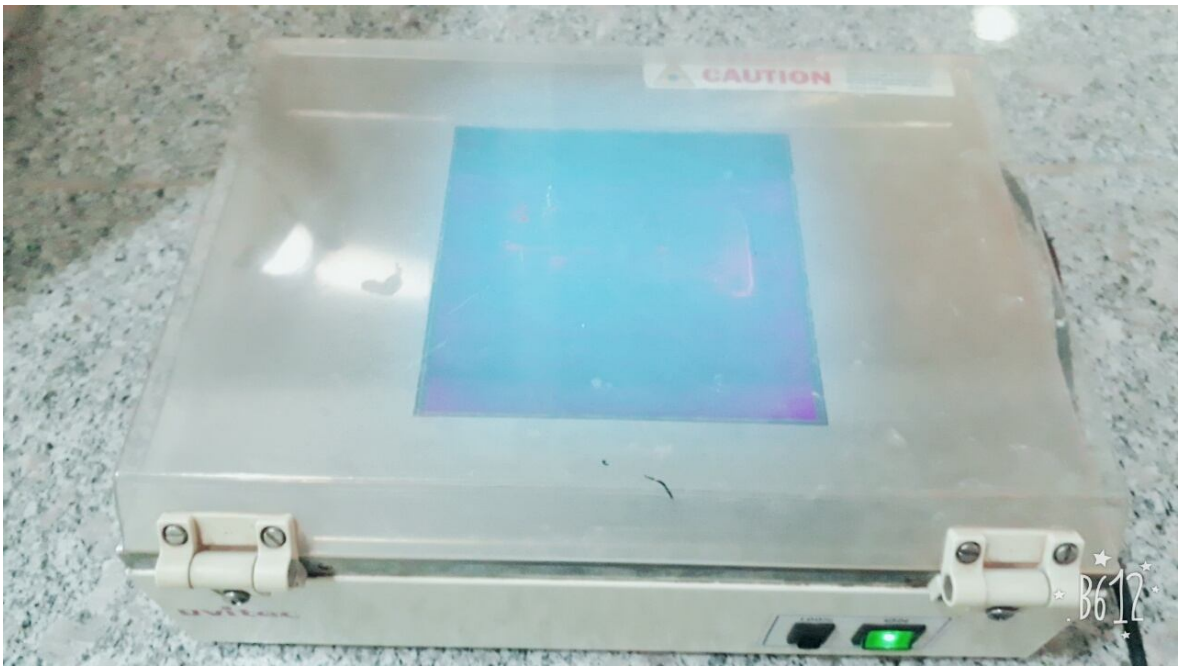

Figure (4) UV Light transilluminater device