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**Phenotypic Detection of Extended-spectrum Beta-lactamases
amongst bacteria isolated from patients in Elmak Nimir
Teaching Hospital - Shendy**

الكشف المظهري عن انزيمات البيتالاكتام واسعة الطيف لدى البكتيريا المعزولة
من المرضى بمستشفى المك نمر التعليمي - شندي

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(سورة المجادلة الآية 11)

DEDICATION

To my parents,

To my teachers,

To my friends and colleagues

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ABSTRACT

The resistance to the antibiotics is a world-wide health problem, Beta-lactam antibiotics are the most common used antibiotics for several systemic infections. Production of these enzymes lead to failure of treatment and longer hospital stay.

The objective of this study was to detect the production of extended-spectrum-beta-lactamases in clinical isolates from Elmak Nimir Teaching Hospital in Shendy.

A total of 100 clinical specimens were collected, these specimens were 67 urine specimen, 18 wound swabs and 15 ear swabs. The specimens were collected from both males and female, and the age range was from 10 to 70 years old. These specimens were cultured on MacConkey agar, mannitol salt agar and cysteine lactose electrolyte deficient (C.L.E.D) to isolate a pure clinical isolates. Then identified according to their colonial morphology, gram reaction and biochemical reactions. These isolates were tested for susceptibility for several antibiotics by disc diffusion technique. And then double disc confirmatory test to detect the production of extended-spectrum-beta-lactamases. These specimens showed an isolation of 66 *E.coli*, 23 *Ps. Aeruginosa*, 8 *K. pneumoniae*, and 3 *S. aureus*. The most frequent organisms from urine specimens were *E. coli* 48/67 (71.6%), *Ps. Aeruginosa* 14/67 (20.8%), *K. pneumoniae* 4/67 (6%), and *S. aureus* 1/67 (1.5%). The most common isolate in the wound swabs were the *Ps. Aeruginosa* 8/18 (44.4%), *E. coli* 7/18 (39%), *K. pneumoniae* 1/18 (5.5%) and the *S. aureus* 2/18 (11%). In the ear swabs the *E. coli* frequent was 11/15 (73%), *Ps. Aeruginosa* frequent was 3/15 (20%) and the *K. pneumoniae* frequent was 1/15 (7%). The isolates were 100% sensitive to impeniem, 92% sensitive to gentamycin, 82% sensitive ciprofloxacin, 77% sensitive to nitrofurantion and 37% sensitive to ceftazidime. 34 out of the 100 isolates were identified as extended-spectrum-beta-lactamases by the double disc confirmatory test.

This study concluded that there is high rate of antibiotic resistance, so the appropriate drug prescriptions by the physicians based on susceptibility testing, and appropriate dose uptake by the patients become necessary to decrease the evolution of antibiotic resistance.

ملخص الأطروحة

إن مشكلة مقاومة المضادات الحيوية هي مشكلة عالمية. مضادات البيتاالاكتام الحيوية هي أكثر أنواع المضادات الحيوية استخداما للعديد من الالتهابات. نتاج إنزيمات البيتاالاكتام يؤدي الى فشل العلاج والبقاء في المستشفى لفترة أطول.

الهدف من هذه الدراسة كان الكشف عن إنتاج إنزيمات البيتاالاكتام واسعة الطيف في العينات السريرية التي تم جمعها من مستشفى الملك نمر التعليمي بشندي.

تم جمع 100 عينة سريرية هذه العينات تم تزييعها على أوساط الماكونكي والسيستين للاكتوز قليلة الشوارد، بيئة أملاح المانيتول وبيئة الدم الغذائية للحصول على معزولات سريرية نقية. تم التعرف على العزلات بناء على شكل المستعمرة، وتفاعلها مع صبغة الغرام والتفاعلات البيوكيميائية. تم اختبار العزلات التي تم الحصول عليها للحساسية للعديد من المضادات الحيوية باستخدام طريقة انتشار القرص، تم الكشف عن إنتاج إنزيمات البيتاالاكتام واسعة الطيف باستخدام طريقة القرص المزدوج التأكيدي. نوع العينات كان يتضمن : 67 عينة بول ، 18 مسحة جروح و 15 مسحة أذن. جمعت هذه العينات من الجنسين ذكور وإناث من مختلف الأعمار وقد كان مدى الأعمار يبدأ من 10 سنوات وحتى 70 سنة. تم عزل 66 باكتريا من الاشرىكية القولونية، 23 من باكتريا الزائفة الزنجارية ، 8 من الكلبسيلا الرئوية و 3 من المكورات العنقودية الذهبية. أكثر الباكتريرات تردد في عينات البول كانت الشريكية القولونية 67/48 (71.6%)، ثم الزائفة الزنجارية 67/14 (20.8%)، والكلبسيلا الرئوية 67/4 (6%) والمكورات العنقودية الذهبية 67/1 (1.5%). في مسحات الجروح ، الزائفة الزنجارية كانت الأكثر ترددا 18/8 بنسبة (44.4%)، الاشرىكية القولونية 18/7 (39%) ، والكلبسيلا الرئوية 18/1 (5.5%) والمكورات العنقودية الذهبية 18/2 (11%). في مسحات الأذن تردد الاشرىكية القولونية كان 15/11 بنسبة (73%)، وتردد الزائفة الزنجارية 15/3 (20%) والكلبسيلا الرئوية 18/1 (7%). هذه العزلات كانت حساسة لمضاد الإيمبيينم بنسبة (100%)، ولمضاد الجينتاميسين بنسبة (92%) ، وبنسبة (82%) لمضاد السبيروفلو كاسسين ، وحساسا لمضاد النايتروفورنتوين بنسبة (77%) وبنسبة (37%) لمضاد السيفتيزبيديم.

34 من أصل 100 من هذه العزلات تم الكشف عن إنتاجها لإنزيمات البيتاالاكتام واسعة الطيف عن طريقة القرص المزدوج التأكيدي.

خلصت هذه الدراسة إلى أن المعدل العالي لمقاومة المضادات الحيوية شائعة الإستخدام، لذلك فإن الوصف المناسب للمضادات الحيوية عن طريق الأطباء اعتمادا على اختبارات الحساسية للمضادات الحيوية ، وأخذ الوصفات والجرعات بالطريقة المثلى بواسطة المرضى أصبحت مهمة لتقليل تطور المقاومة ضد المضادات الحيوية.

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CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1. Introduction :

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

Members of enterobacteriaceae including *klebsiella pneuminae* (*K. pneuminae*), *Eschrichia coli* (*E.coli*), as well as *Enterobacter* sp. As well as non lactose ferementing bacteria such as *Pseudomonas aeruginosa* (*Ps. aeruginosa*), and *Actinobacter* sp. Have been identified as major cause of multi drug resistant bacterial infections (Rossolini *et al.*, 2015). These resistance among Gram-negative bacteria to commonly used antibiotics, leading to loss of efficacy for treatment of common infections (Le doare *et al.*, 2015). The resistant bacterial pathogens are major cause of both community and hospital-acquired infections. Respiratory tract, urinary tract , blood stream (septic), post-surgical (wound) infections and pneumonia are among most commonly reported infections attributed to these pathogens in many hospitals (Allegranzi *et al.*, 2011).

Beta lactamases are enzymes produced by some bacteria and are responsible for resistance to beta-lactam group of antibiotics like penicillin, cephamycin and carbapenems (Bush *et al.*, 1995). Some of these enzymes include extended-spectrum-beta-lactamses (ESBL), AmpC beta-lactamases (AmpC BL) and carbapenemase. ESBL are the plasmid mediated beta lactamases that are capable of efficiently hydrolyzing penicillin, narrow and broad spectrum cepahlosporins and monobactams, but they do not hydrolyze cephamycin or carbapenemase (Emery and Weymouth, 1997).

Beta lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam generally inhibit ESBL producing strains (Naumorskii and palzkii, 1997). Extended-spectrum-beta-lactamases are commonly found in *K. pneumoniae* and *E. coli* (Winkour and Bruggemann-Desalvo, 2005). AmpC BL are primarily chromosomal and plasmid mediated and are resistant to beta lactamases inhibitors such as clavulanic acid but can hydrolyze cephamicin (Livermore and Woodford, 2000). Carbapenemase are one of antibiotics of last resort of many bacterial infections such as *E. coli* and *K. pneumoniae* producing AmpCs and ESBLs but the emergence of carbapenems which have versatile hydrolytic capacities have the ability to hydrolyze penicillins, cephalosporins, monobactams and carbapenems (Fernando *et al.*,2009). There are different methods for detection of beta-lactamases including: disc diffusion method, double disc approximation method, this method is based on synergy between amoxicillin-clavulanate and a third generation cephalosporins placed at a distance of 20 mm from center to center on Muller-Hinton agar plate swabbed with the test isolate,clear extension toward the cephalosporins disc reported as positive for ESBL production. Also many molecular techniques can be used as polymerase chain reaction which is based on amplification of the target sequence. There were many studies carried in Sudanese patients from UTI and the results showed that 55 out of 71 *E. coli* and 95 *K. pneumoniae* ESBL producing organisms. ESBL genes were detected in 55 species (50.5%) of *K. pneumoniae* and 40 species (56.3%) of *E. coli*. The most common genotype was bla CTX-M(46%), followed by bla TEM (30.2%) and bla SHV(23.8%) (Yousif *et al.*, 2016).

1.2. Rationale

The antibiotic resistance is a world-wide health problem. It lead to more of failure in treatment for bacterial infections. Beta-lactam antibiotics are most common drugs used for several infections. In the last years many studies showed that there is a high rate of treatment failure in different states of Sudan (Mekki *et al.*, 2010). This study aims to detect the production of extended-spectrum-beta-lactamases in different clinical isolates in Shendy, Nahr Elneel state.

1.3. Objectives

1.3.1. General objective

To phenotypic detection of extended-spectrum-beta-lactamases from different clinical Isolates.

1.3.2. Specific objectives

1. To isolate and identify bacteria from different clinical isolates.
2. To perform antimicrobial susceptibility test.
3. To perform double disc confirmatory technique to detect the presence of extended-spectrum-beta-lactamases.

CHAPTER TWO

LITERATURE REVIEW

2.1. Antibiotics

Are natural, synthetic or semi synthetic compounds use for treatment of bacterial infections; they act either by killing or inhibit the growth of bacteria. The antibiotics have been divided into different families according to their chemical structure, mode of action and the site of action (Fisher *et al.*, 2005).

2.2.1. Beta-lactam antibiotics

Beta-lactam antibiotics are a class of broad spectrum antibiotics, consisting of all antibiotics that contain a beta-lactam ring in their molecular structures, this include penicillin derivatives (penems), cephalosporins (cephems), monobactams and carbapenems (Holten and Onusko, 2000). Beta-lactam antibiotics are bactericidal and act by inhibiting the synthesis of peptidoglycan layer of bacterial cell walls, the peptidoglycan is important for the cell wall integrity, especially in Gram-positive organisms (Fisher *et al.*, 2005).

2.2.2. Resistance to beta-lactam

The bacteria can develop a resistance against the beta-lactam group of antibiotics by production of the enzyme beta-lactamases, these enzymes hydrolyses the beta-lactam ring of the antibiotic rendering the antibiotic ineffective (Leonard *et al .*, 2013). Beta-lactamases deactivates the molecular antibacterial properties there by breaking and opening their common element in their molecular structure beta-lactam (Bush *et al.*, 1995). The production of beta-lactamases by a bacterium does not necessarily rule out all treatment options with beta-lactam antibiotics. In some instances , beta-lactam antibiotics may be co-administrated with beta-lactamases inhibitor. For example, Augmentin (FGP) is made of amoxicillin(beta-lactam antibiotic) and calvulanic acid (beta-lactamases inhibitor) the calvulanic acid is designed to overwhelm all beta-lactamase enzymes, and effectively serve as antagonist so that the amoxicillin is not affected by beta-lactamase enzymes. Other beta-lactamases inhibitors such as boronic acid are being studied in

which they are irreversibly bind to the active site of beta-lactamases. This benefit over clavulanic acid and similar beta-lactamases competitors, because they cannot hydrolyzed, and there for rendered useless. Extensive research is currently being done to develop tailored boronic acid to target different isoenzymes of beta-lactamases (Leonard *et al.*, 2013).

2.3. Types of beta-lactamases

2.3.1. Penicillinase

Is a specific type of beta-lactamases showing specificity for penicillins, again by hydrolyzing the beta-lactam ring. Molecular weight of the various penicillinases tend to cluster near 50 kilodaltons. Penicillinase was the first beta-lactamase to be identified, it was first isolated by Abraham and Chain in 1940 from Gram-negative *E. coli* even before penicillin entered clinical use (Abraham and Chain, 1940). Members of the family commonly express plasmid-encoded beta-lactamases (e.g. TEM-1, TEM-2, and SHV-1). Which confer resistance to penicillinase but not to expanded-spectrum cephalosporins. In the mid of 1980's, a new group of enzymes, the extended-spectrum-beta-lactamases was detected (Bush *et al.*, 1995).

2.3.2 TEM beta-lactamases

(class A) TEM-1 is the most commonly encountered beta-lactamases in Gram-negative bacteria, up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Paterson *et al.*, 2003). The term TEM comes of the Athenian patient (Temoniera) from which the isolate was recovered in 1963 (Yousif *et al.*, 2018).

2.3.3. SHV beta-lactamases

(class A) shares 68 percent of its amino acids with TEM-1 and has similar overall structure. The SHV-1 beta-lactamases is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid mediated ampicillin resistance in this species. ESBLs inhibits family also have amino acids changes around the active site, most commonly at positions 238 or 240. More

than 60 SHV varieties are known. SHV-5 and SHV-12 are among the most common (Paterson *et al.*, 2003).

2.3.4. CTX-M beta-lactamases

(class A) these enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrate. These enzymes are not very closely related to TEM and SHV beta-lactamases in that they show only 40% identity with these two commonly isolated beta-lactamases (Fisher *et al.*, 2006).

2.4 Classification of ESBLs

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

Classification scheme for beta-lactamases proposed by Ambler has found common usage (Ambler *et al.*, 1991; Ambler, 1980). Ambler classifies these enzymes into four classes, A, B, C and D (Ambler, 1980; Jaurin and Grundstrom, 1981; Ouellette *et al.*, 1987). Whereas classes A, C and D have evolved dependence on an active-site serine as their key mechanistic feature, class B enzymes are zinc dependent and hence different. The catalytic process for turnover of the members of the former group involves acylation at active-site serine by the beta-lactam antibiotic, followed by deacylation of the acyl-enzyme species. It is noteworthy that these enzymes do not share any sequence homologies, structural similarities, or mechanistic features with serine or zinc-dependent proteases. Class A beta-lactamases generally prefer penicillins as substrates, whereas class C enzymes turnover cephalosporins better. Class B enzymes can hydrolyze a broad range of substrates including carbapenemase, which resist hydrolysis by most of the other classes of enzymes. Class D beta-lactamases, on the other hand, hydrolyze oxacillin type beta-lactams efficiently. Classes A and C of beta-lactamases are the most common and the second most common enzymes, respectively (Baciero and Bou, 2004; Yang *et al.*, 1999; Bush and Mobashery, 1998).

According to the Bush and Jacoby scheme (Bush and Jacoby, 2010) ESBLs enzymes can be divided into three main groups: group 1 cephalosporinases which are not inhibited by clavulanate.

Group 2 broad spectrum enzymes which comprise the largest group and are generally inhibited by clavulanate except the 2d and the 2f groups (Dhillon and Clark, 2012). Subgroups of enzymes, namely 2a, 2b, 2be, 2br, 2c, 2d, 2e and 2f, were defined based on the rates of hydrolyses of carbenicillin, cloxacillin, extended-spectrum-beta-lactamases ceftazidime, cefotaxime or aztreonam, and on inhibition profile by clavulanate, respectively. Enzymes that are inhibited by the metal-chelating agent EDTA are classified as group 3. Group 4 consists of other beta-lactamases that are not inhibited by clavulanic acid (Ambler *et al.*, 1991; Ambler, 1980). However, most ESBLs are allocated to group 2be which can hydrolyze penicillins, monobactams and cephalosporins, and are inhibited by clavulanate, they are class A according to the Ambler molecular scheme. CTX-M genotype still suits the former criteria for group 1be enzymes (Dhillon and Clark, 2012).

2.4.1 Class A beta-lactamases

Generally, class A beta-lactamases enzymes are susceptible to beta-lactamases inhibitors such as clavulanate, tazobactam and to a lesser extent sulbactam. However the *K. pneumoniae* carbapenemase KPC may be an exception to this generalization (Papp-Wallace *et al.*, 2009). The first plasmid mediated member of this class was described in *Escherichia Coli* in 1963, and was named TEM (Datta and Kontomichalou, 1965). SHV which is another common beta-lactamases detected primarily in *K. pneumoniae* (Matthew *et al.*, 1979). TEM and SHV are common beta-lactamases described in clinical isolates of *E. coli* and *K. pneumoniae*, primarily responsible for urinary tract, blood stream and hospital acquired respiratory tract infections (Buynak, 2006).

2.4.2 AmpC type beta-lactamases (Class C)

AmpC type beta-lactamases are commonly isolated from extended-spectrum-cephalosporins-resistant Gram-negative bacteria. AmpC beta-lactamases (also termed class C or group 1) are typically encoded on the chromosome of many Gram-negative bacteria including *Citrobacter*, *Serratia* and *Enterobacter* species where it's expression is usually inducible, although it can be hyper expressed. AmpC type beta-lactamases may also be carried on plasmids (Yasmin, 2012).

2.4.3. OXA beta-lactamases (Class D)

OXA beta-lactamases were long recognized as a less common but also plasmid mediated beta-lactamases 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 (Yasmin, 2012).

2.4.4. Others

Other plasmid mediated ESBLs, such as PER, VEB, GES and IBS beta-lactamases, have been described but are uncommon and have been found mainly in *Ps. aeruginosa* and at a limited number of geographic sites. PER-1 in isolates in Turkey, France and Italy; VEB-1 and VEB-2 in strains from southeast Asia; and GES-1, GES-2 AND IBC-2 in isolates from South Africa, France and Greece (Yasmin, 2012).

2.5 Epidemiology

The epidemiology of ESBLs is quite complicated. Initially, there are certain levels to consider: the wider geographical area, the country, the hospital, the community and the host (in most cases a single patient or a healthy carrier). Moreover, these are bacteria (*E. coli* is more endemic and *K. pneumoniae* is more epidemic) and their mobile genetic elements, usually plasmids. Additionally, there are various reservoirs, including the environment (e.g. soil and water), wild animals, farm animals and pets. The final component entails transmission from food and water, and via direct or indirect contact (person to person) (Carroti, 2008). The first ESBL to be identified was found in Germany in 1983, but it was in France in 1985 and in the United States at the end of 1980's and the beginning of 1990's that the initial outbreaks occurred (Rice *et al.*, 1990). New TEM and the SHV enzymes are still emerging in Europe, and distinct epidemic clones have been found, for example *Salmonella* isolates with TEM-52 in Spain (Fernandez *et al.*, 2006) and *E. coli* isolates with SHV-12 in Italy (Pereilli *et al.*, 2011). Isolates with the CTX-M-9 group are common in Spain and strains with the CTX-M-3 enzymes have been described

chiefly in western Europe, although clones producing the CTX-M group-1 (including the CTX-M-15 type) are the most widespread throughout Europe (Coque *et al.*, 2008a,b; Canton *et al.*, 2008). Today, *E. coli* and the CTX-M enzymes are not uncommon in outpatients (Aktas *et al.*, 2008).

A study conducted at the national public health laboratory (NPHL), Kathmandu, Nepal reported that (31.57%) of *E. coli* were confirmed as extended-spectrum-beta-lactamases producers, these isolates further exhibited co-resistance to several antibiotics (Thakur *et al.*, 2013). The overall data on ESBL-producing Enterobacteriaceae in the countries of the Middle East are extremely worrisome, and this region might indeed be one of the major epicenters of the global ESBL pandemic (Mohamed AlAgamy *et al.*, 2006). Investigation conducted in Saudi Arabia in 2004-2005 showed that (10%) of clinical urinary *E. coli* isolates from inpatients and 4% of such isolates from outpatients were ESBL producers (Khanfar *et al.*, 2009). Moubareck and colleagues analyzed fecal samples Lebanon in 2003 and noted that ESBL carriage differed somewhat between patients (16%), healthcare workers (3%) and healthy subjects (2%), and also that there was a predominance of the CTX-M-15 enzyme (83%) (Moubareck *et al.*, 2005). Other researchers in Lebanon (Khanfar *et al.*, 2009) observed that the proportion of ESBL-producing isolates was significantly larger among inpatients (15.4%) than in outpatients (4.5%). Moreover, data collected over three years in Kuwait showed that the level of ESBLs were lower in community isolates of *E. coli* (12%) than in the corresponding hospital isolates (26%) (AlBenwan *et al.*, 2010). An investigation of 10 other centers in India showed that rates of ESBL producing Enterobacteriaceae reached (70%) (Mathai *et al.*, 2002). Recently ESBL production was observed in (48%) of *E. coli* isolates in a tertiary hospital in Patiala, Punjab (Rupinder *et al.*, 2013). In other recent studies, authors observed ESBL rates of (46%) and (50%) in out- and inpatients, respectively (Sanker *et al.*, 2012), and Nasa and co-workers detect ESBL production in almost (80%) of clinical isolates (Nasa *et al.*, 2012). Investigations from India and Pakistan show an alarming increase in prevalence of Enterobacteriaceae with NDM-1 with prevalence rate from (6.9%) in a hospital in Varanasi, India, to (18.5%) in Rawalpindi, Pakistan (Perry *et al.*, 2011; Majda *et al.*, 2013), reported that (72%) of *E. coli* were ESBL producers at the Microbiology Laboratory of Shalamar Medical College, Lahore. Sensitivity testing showed a multi-drug resistance in ESBL producing *E. coli*. Maximum resistance was recorded in *E. coli* (ESBL) as cefotaxime (98.8%), Ceftazidime (96.7%), and Cefuroxime (93.4%) while minimum

resistance was seen Imipenem (0.8%), Fosfomycin (1.2%) and Nitrofurantion as well as Piperacillin/Tazobactam (2.2%) (Gerasimovska and Gerasimovska-Kitanovska, 2015), performed a Study included 80 patients, 40 with ESBL positive and 40 with ESBL negative strains of *E. coli*. The groups did not differ in number, gender and age, but ESBL positive groups was of older age and had more case with urosepsis than ESBL negative group. Regarding the morbidities, most of the patients had diabetes mellitus type 2 in 16 patients (40%) and 15 of them (37%) had chronic kidney disease. In most of the patients, antibiotics were used empirically before urine culture was obtained, which accounts for high number of cephalosporins and aminoglycosides used-9 patients (22%), and Amikacin in 11 (27%), Imipenem (8%). Univariate analysis of risk factors associated with ESBL + infections. When the three variables were entered into a multivariate logistic regression, diabetes and sepsis were found to be predictors of ESBL positive patients.

Also (Tabar *et al.*, 2016), in Semnan, Iran collected 2618 urine specimens, one hundred nine samples were identified as *E. coli* (4.16%). Twenty one (26.6%) of *E. coli* were ESBL positive and (73.4%) were ESBL negative. Twenty six (89.65%) of the positive samples were female and three (10.34%) were male. The average age was 32 years old and patient age ranged from 6 days to 87 years old. There was 100% susceptible to Imipenem, Twenty (68.97%) out of 29 isolates were positive for the CTX-M gene as detected by PCR (Mekki *et al.*, 2010) in Khartoum isolated hundred strains of multi drug resistant *E. coli* and *Klebsiella* species causing nosocomial UTI from two main hospitals from Khartoum (Omdurman teaching hospital and Fedail hospital). Susceptibility testing was performed against antibiotics commonly used in treatment of UTI. *E. coli* was among the study isolates. Beta-lactamases was produced by most of isolates; high resistance level for third generation cephalosporin was noticed. ESBLs were detected in high prevalence among all multi-drug resistant *E. coli* and *Klebsiella* species isolates (53%) (Vieyra *et al.*, 2016). Isolated 404 non-repeated positive ESBL *E. coli* which were collected from documented clinical infections in pediatric patients over a 2-years period in Mexico. Isolates that suggested ESBL production based on their resistance profiles to third and fourth generation cephalosporin and monobactam were selected. ESBL production was phenotypically confirmed using a diffusion method with cefotaxime and ceftazidime discs alone and in combination with calvulanic acid. Bla ESBL gene identification was performed through PCR amplification and sequencing pulsed field Gel Electrophoresis (PFGE) and multilocus sequence typing (MLST)

were detected in (2.5%) of the isolates(Hernande\ *et al.*, 2005). In Spain made a clonal dissemination of ESBL in 170 *E. coli* isolates from a nation-wide study of 40 Spanish centers. The most prevalent ESBL were CTX-M-9 (27.3%), SHV-12(23.9%) and CTX-M-14 (20.4) for *E. coli* (Oteo *et al.*, 2006). Isolated 151 *E. coli* strains resistant to cefotaxime and ceftazidime during a prospective surveillance study. These were characterized by clinical, microbiological and molecular analysis and were distributed into four clusters of 103, 11,6 and 5 isolates, along with 25 unrelated strains. The principle cluster was isolated from urine,wound,blood and other samples in three hospitals, eight nursing homes, and community health care center. This cluster was associated with both nosocomial (65%) and community acquired (35%) infections. All isolates from the four clusters expressed the ESBL CTX-M-15. This enzyme was also present in 8 (30.8%) of the 26 unrelated isolates. The other ESBLs, CTX-M-14 and CTX-M-32 were detected in five and seven cases (Saeide *et al.*, 2014), in Zabol, Iran collect ninety isolates of *E. coli* from urinary tract infection, The results of disc diffusion method that forty out of ninety *E. coli* isolates were ESBL producing organisms. Antibiotic susceptibility of *E. coli* to 9 antibacterial agents was evaluated. However, all *E. coli* isolated were resistant to all 9 antibacterial agents by this percentage: ceftriaxone (100%), ceftazidime (100%), amoxicillin (100%), erythromycin (100%), cefixime (87.5%), tetracycline (87.5%), naldixic acid (85%) and difloxacin (75%). The abundance of antibiotic-resistant TEM gene according to PCR was (30%). Totally (82.5%) of strains were observed as ceftazidime resistant (Omati *et al.*, 2016). Isolated in the city of Sanandaj 180 *E. coli* strains from urine. 89 samples were ESBL-positive. PCR assay used for detecting the CTX-M gene, showed that 48 samples out of 180 samples (26.66%) contained that gene; also among these 48 samples, 23 (12.77%) had CTX-M group 1 based on the REP-PCR assay; all CTX-M producing species are not originated from one single strain and the gene is spread between different isolates (Omati *et al.*, 2016).

CHAPTER ONE

MATERIALS AND METHODS

3.1. Type of study

This is a cross-sectional study.

3.2. Study population

The study was done on 100 patients with different bacterial infections to determine the frequency of the extended-spectrum-beta-lactamases producing isolates among the different clinical isolates. The study population included both male and female, from all ages and the age range was from 10 to 70 years.

3.3. Study area

The study was conducted in Elmak Nimir Teaching Hospital in Shendy.

3.4. Sample size

The totals of 100 patients were enrolled in this study.

3.5. Ethical consideration

A verbal consent was obtained from each patients participated in this study.

3.6. Collection of specimens

Different types of specimens were collected. This include: 67 urine specimens, 15 ear swabs and 18 wound swabs.

3.7. Isolation of causative agents

Different types of culture media including: MacConkey agar, mannitol salt agar, blood agar and cysteine lactose electrolyte deficient (C.L.E.D) were used to obtain a pure isolates. The urine specimens were inoculated on the plates by using a wire loop, a drop of mid-stream urine was placed on the plates of (C.L.E.D) and MacConkey agr and then distributed on the surface of plate to obtain a single colony, then incubated at 37°C for 18-24 hrs. After incubation the pure isolated colonies were tested for colonial morphology and lactose fermentation. The wound and ear swabs were cultured on blood agar, MacConkey agar and mannitol salt agar and incubated at 37°C for 18-24 hrs. After incubation the pure isolated colonies were tested for colonial morphology.

3.7.1. Identification technique

3.7.1.1. Gram staining technique

3.7.1.2. Smear preparation

The pure colonies were emulsified in a drop of distilled water in clean dry microscopic slide and allowed to air dry then dried smear was fixed by passing three times over the flame (Collee and Jhono, 2002).

3.7.1.1.2. Staining procedure

The fixed smear was covered with crystal violet solution for one minute. The smear was washed with tab water and covered with Gram iodine solution for one minute then washed with acetone alcohol solution for seconds then washed with tab water. The smear was covered with safranine solution for two minutes, then washed with tab water and allowed to air dry. The smear was examined with oil immersion lens (Collee and Jhono, 2002).

3.7.2.1. Kligar Iron Agar

The kligar iron agar medium was used to detect the lactose fermentation, glucose fermentation, hydrogen sulphide production and gas production. The test was performed by stabbing the tested organism into the butt first and then a zigzag pattern on the slope. Then the inoculated medium

was incubated aerobically at 37°C for 18-24 hrs. the result was interpreted as follow: a red slope with a yellow butt indicates fermentation of glucose only, a yellow slope with yellow butt indicates fermentation of both glucose and lactose, a red slope with red butt indicates no fermentation happened. Crack and bubbles on media indicates gas production. Black color indicates hydrogen sulphide production (Cheesbrough, 2006).

3.7.2.2. Citrate utilization test

The test was performed by inoculating the tested organism on Simmon's citrate agar on a slope medium then incubated for up to 4 days at 37°C. the bromothymol blue indicator turn the media from green to blue in a positive result and there should be observable growth on slope (Tille, 2014).

3.7.2.3. Indole test

The test was performed by inoculating the tested organism on a tube of sterile peptone water contain tryptophan by wire loop; incubated at 37°C for 18-24 hrs. After incubation 0.5 ml of Kovac's reagent was added to examined the production of red color within 10 seconds is considered positive (Tille, 2014).

3.7.2.4. Urease test

The test was performed by inoculating the tested organism on slope surface of Christensen's urea agar then incubated for overnight at 37°C. A red pink color is considered positive (Tille, 2014).

3.7.2.5. Methyl red test

The test was performed by inoculating the tested organism on glucose phosphate peptone water (0.5ml) after overnight incubation at 37°C a drops of methyl red solutions were added the appearance of red color indicates a positive result, and the yellow color indicates negative result. (Tille, 2014).

3.7.2.6. Motility test

The test was performed by inoculating the tested organism on a semi-solid media stabbed to depth of only one third inch in the middle of the tube, the tube was incubated at 37°C for 18-24 hrs. Motile organisms spreaded out into medium from the site of inoculation, while non-motile organisms remain at the site of inoculation (Tille, 2014).

3.8. Antimicrobial susceptibility test

After isolation of the organisms they were examined to determine their susceptibility to antibiotics which included (Imipenem, Gentamycin, Nitrofruntion, Cefatizdime and Ciprofloxacin). Muller-Hinton agar was prepared and antibiotics discs provided from Hi Media for medical products were used, an organism suspension matched it's turbidity with a 0.5 Mc Farland standard was prepared, then the plate was selled by the tested organism and the discs were placed. The plate was incubated at 37°C for 18-24 hrs, after incubation the inhibition zone was measured to determine it with provided chart (Chessbrough, 2006).

3.9. Double disc confirmatory test

The double disc confirmatory test was carried on the resistant isolates. Amoxicillin/clavulnic acid (20/10µg) disc was placed in the center of the Muller-Hinton agar on which a lawn culture of the test organism was prepared, cefatizidme (CAZ 30µg) and cefotaxime (CTX 30µg) were placed on either side of the plate. Plates were incubated for 18-24 hrs at 37°C, and the pattern of inhibition was noted. Isolates that exhibited a potentiating towards amoxicillin/calvulanic acid were considered potential ESBL producer (Tille, 2014).

3.10. Data analysis

The data was analyzed by using the statistical packaged for social science (SPSS) version 16 by using Chi-square test to determine the significance of data (P value <0.5 were considered significant).

CHAPTER FOUR

RESULTS

A total of 100 clinical specimens were collected from Elmak Nimir Teaching Hospital. The specimens were collected from both genders and with an age range from 10 to 70 years.

67 urine specimens, 15 ear swabs and 18 wound swabs. Identification techniques were performed on these specimens.

The results showed an isolation of 66 *E. coli*, 23 *Ps.aeruginosa*, 8 *K. pneumoniae* and 3 *S. aureus*. 48 isolates of *E. coli* were obtained from the urine specimens, 7 from wound swabs and 11 from the ear swabs. 14 isolate of *Ps.aeruginosa* were obtained from urine specimens, 8 from wound swabs and 1 from ear swabs. 4 isolates of *K. pneumoniae* were obtained from urine specimens, 1 from wound swabs and 3 from ear swabs. 2 isolates of *S. aureus* were obtained from urine specimens and 1 from the wound swabs as shown in table (4) and table (5).

Then the antimicrobial susceptibility test was performed by using Imipenem, Gentamycin, Nitrofruntion, Cefatizdime and Ciprofloxcain discs the isolates showed (100%) sensitive to nitrofurantion and 37% sensitive to ceftazidime as shown in table (6).

The isolates were tested for extended-spectrum-beta-lactamases (ESBL) production by the double disc confirmatory result showed 34% of isolates are ESBL producers. The isolates showed varied results, *E. coli* 24 out of 66 were identified as ESBL producers (36%), *Ps. aeruginosa* 9 out of 23 were identified as ESBL producers.

Table (1) Distribution of different clinical specimens according to the gender

| Specimen | Male | Female | Total |
|-------------------|-------------|---------------|--------------|
| Urine | 49 | 18 | 67 |
| Wound swab | 12 | 06 | 18 |
| Ear swab | 07 | 08 | 15 |
| Total | 68 | 32 | 100 |

Table (2) Distribution of different clinical specimens according to the age groups

| Specimen | (10-25) | (26-40) | (41-55) | (56-70) |
|-------------------|----------------|----------------|----------------|----------------|
| Urine | 9 | 36 | 12 | 10 |
| Wound swab | 5 | 5 | 6 | 2 |
| Ear swab | 3 | 2 | 3 | 7 |
| Total | 17 | 43 | 21 | 19 |

Table (3) Frequency of different clinical specimens

| Specimen | Frequency |
|-----------------|------------------|
| Urine | 67 |
| Wound swab | 18 |
| Ear swab | 15 |

Table (4) Frequency of different clinical isolates

| Isolate | Frequency |
|-----------------------|------------------|
| <i>E. coli</i> | 66 |
| <i>Ps. aeruginosa</i> | 23 |
| <i>K. pneumoniae</i> | 8 |
| <i>S. aureus</i> | 3 |
| Total | 100 |

Table (5) Distribution of different clinical isolates among the clinical specimens

| Isolate | Urine | Wound swab | Ear swab |
|-----------------------|--------------|-------------------|-----------------|
| <i>E. coli</i> | 48 | 7 | 11 |
| <i>Ps. aeruginosa</i> | 14 | 8 | 1 |
| <i>K. pneumoniae</i> | 4 | 1 | 3 |
| <i>S. aureus</i> | 1 | 2 | - |

Table (6) Susceptibility to the antibiotic discs among different clinical isolates

| | Ic | | Gen | | Caz | | Nit | | Cip | | |
|-----------------------|-----------|----------|------------|----------|------------|----------|------------|----------|------------|----------|--|
| Isolate | S | R | S | R | S | R | S | R | S | R | |
| <i>E. coli</i> | 66 | 0 | 61 | 3 | 21 | 45 | 57 | 9 | 55 | 11 | |
| <i>Ps. aeruginosa</i> | 23 | 0 | 21 | 2 | 9 | 14 | 12 | 11 | 19 | 4 | |
| <i>K. pneumoniae</i> | 8 | 0 | 5 | 3 | 4 | 4 | 6 | 2 | 5 | 3 | |
| <i>S. aureus</i> | 3 | 0 | 3 | 0 | 3 | 0 | 2 | 1 | 3 | 0 | |
| | 100 | 0 | 92 | 8 | 37 | 63 | 77 | 23 | 82 | 18 | |

* IC ≡ imipenem, Gen ≡ gentamycin, Caz ≡ cefatzidime, Nit ≡ nitrofurantoin,

Cip ≡ ciprofloxacin

Table (7) Distribution of ESBL production among different clinical isolates

| Isolate | ESBL positive | ESBL negative | Total |
|-----------------------|----------------------|----------------------|--------------|
| <i>E. coli</i> | 24 | 42 | 66 |
| <i>Ps. aeruginosa</i> | 9 | 14 | 23 |
| <i>K. pneumoniae</i> | 1 | 7 | 8 |
| <i>S. aureus</i> | 0 | 3 | 3 |
| Total | 34 | 66 | 100 |

Table (8) Distribution of ESBL production according to gender

| Gender | ESBL positive | ESBL negative | Total |
|---------------|----------------------|----------------------|--------------|
| Male | 26 (38%) | 42 (62%) | 68 |
| Female | 8 (25%) | 24 (75%) | 32 |
| Total | 34 | 66 | 100 |

P value = 0.652

Table (9) Distribution of ESBL production according to age groups

| Age group | Positive ESBL | Negative ESBL | Total |
|------------------|----------------------|----------------------|--------------|
| 10 – 25 | 6 | 11 | 17 |
| 26 – 40 | 17 | 27 | 44 |
| 41 - 55 | 5 | 16 | 21 |
| 56 – 70 | 6 | 12 | 18 |
| Total | 34 | 64 | 100 |

P value = 0.512

CHAPTER FIVE

DISCUSSION

The resistance to the third generation cephalosporins has become a cause of concern especially among Enterobacteriaceae which cause non nosocomial infections. The prevalence of ESBLs among the members of Enterobacteriaceae constitute a serious threat to current beta-lactam therapy leading to treatment failure. Among the wide array of antibiotics, beta-lactam antibiotics are the most widely used for over 50% of all the systemic infections (Kumar *et al.*, 2006).

This study demonstrated the presence of ESBL-mediated resistance in different clinical isolates of patients in Elmak Nimir Teaching Hospital in Shendy. The frequency of ESBL-producing isolates was found (34%). This figure is high when compared to the findings 28.2% reported in Tanzania Nduglie *et al.*, (2005), but lower than the finding (53%) in the study carried out in the Khartoum state Mekki *et al.*, (2010). But much higher than the (6.5%) reported in Saudi Arabia Kader and Kumar., (2004). But much lower than the (60.9) observed in Egypt Mohamed-Alagmy *et al.*, (2004), and (78%) in Pakistan Hussein *et al.*, (2011). Overall these findings indicate that the prevalence of ESBL producers isolates is a world-wide health problem, and these showed a variation in the results in the ESBL production =results due to the variation in the sample size, geographical areas and methods of detection. In this study there was insignificant difference observed among different age groups, a previous study by Moyo *et al.*, (2010) reported significantly higher ESBL production in isolates from children rather than adults. This study showed insignificant difference in the production of ESBL between both genders in agreement with study of Akanbi *et al.*, (2013).

5.2. Conclusion

The study showed a high prevalence of ESBL production among the *Ps. aeruginosa* and *E. coli* and *K. pneumoniae* from the different clinical specimen, so to solve this issue the knowledge of pathogenesis, antibiotic resistance mechanism and treatment of causative agents is important, and the antimicrobial susceptibility testing is important to avoid the resistant antibiotic uptake.

5.3. Recommendations

From the results, the study recommended that :

- 1- Culture and susceptibility technique must be performed to determine the appropriate treatment.
- 2- Performing the polymerase chain reaction technique to determine the responsible gene for ESBL production as routine.
- 3- Using more sensitive test to detect the ESBL production.
- 4- The health education for the community about the danger of using antibiotics without medical prescriptions and the danger of the resistance development.
- 5- Continuous studies about the antibiotic resistance because of rapid bacterial evolution.

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