Detection of extended spectrum-beta lactamase enzymes producing *E. coli* that isolated from urine

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Abstract:

The production of extended-spectrum- β lactamases (ESBLs) is an important mechanism for resistance to the third-generation cephalosporins. ESBLs represent a major group of lactamases enzymes that mostly produced by gram-negative bacteria, so the detection of these enzymes are very important for optimal patients care. The present study was done to detect extended spectrum beta lactamase producing E. coli among urinary tract infected patients. A total of 223 urine samples were examined for presence of E. coli and those producing ESBL enzymes. Urine samples were cultured for aerobic bacteria and antimicrobial susceptibility testing carried out by using Kirby-Baur agar diffusion method. Coli were tested for ESBLs on Mueller-Hinton agar by both modified double disk (MDDT) and phenotypic confirmatory test. E. coli was the most common bacteria isolated from urine 104 (44.2).78 E. coli isolated from urine are tested for ESBL production and it was found that 30 (38.4) were MDDT positive and 27 phenotypic confirmatory test positive. Three strain *E. coli* were MDDT positive but negative by phenotypic confirmatory. Antibiotic susceptibility test showed that E. coli isolated were totally resist (100%) to ampicillin, moxicillin,and trimethoprim but maximum susceptible to imipenem (100%) and variable resistant to another antibiotics. The ESBLs producing E. coli are highly resist to different types of antibiotics , especially third generation cephalosporins.

تحديد العزلات المنتجة لانزيمات بيتا لاكتام واسعة الطيف لبكتريا Escherichia Coli المعزولة من الادرار

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الخلاصة:

يعد إنتاج إنزيمات(β- lactamases) واسعة الطيف من ميكانيكيات المقاومة المهمة للجيل الثالث من السفالوسبورين. فهذ الإنزيمات تمثل مجموعة رئيسية من إنزيمات (β- lactamases)المشخصة في عدد كبير من بلدان العالم والتي تنتج بشكل رئيسي من قبل البكتريا السالبة لصبغة كرام .فالتعرف على هذه الإنزيمات مهم جدا لتوفير عناية مثلى للمرضى . تهدف الدراسة الحالية لتحديد إنزيمات (β- lactamases) واسعة الطيف المنتجة من قبل بكتريا القولون(*E. coli*) المعزولة من المرضى

المصابين بالتهاب المجاري البولية.تم جمع وفحص 223 عينة إدرار للتحري عن وجود بكتريا القولون (*E.coli*) وتحديد المنتجة منها لإنزيمات (β- lactamases) واسعة الطيف .ثم زرع عينات الإدرار هوائيا واجراء اختبار فحص حساسية البكتريا لمضادات الحياة وفقا لطريق Kirby-baur وقد استخدام وسط Mueller-Hinton الصلب لتحديد عزلات *E.coli* المنتجة لإنزيمات (β- lactamases) وذلك باستخدام طريقة (Modified double disk) وفحص (Phenotypic confirmatory test) . لقد كانت بكتريا *E.coli* البكتريا السائدة التي تم عزلها من عينات الإدرار إذ تم عزل 104 ونسبة (44.2) من البكتريا المعزولة وقد اختبرت 78 من عزلات *E.coli* المعزولة من عينات الإدرار لتحديد إنتاجيتها لإنزيمات(β- lactamases) واسعة الطيف إذ وجد إن 30 (38.4 %)من العزلات التي تم اختبارها منتجة ل(ESBL) كما كانت كلها موجبة لفحص MDDT ،كما كان منها27 موجبة لفحص (Phenotypic confirmatory test) ،إلا إن ثلاثا من عزلات *E.coli* التي كانت موجبة لفحصMDDT كانت سالبه لفحص (Phenotypic confirmatory test).إجمالا اظهر فحص حساسية بكتريا *E.coli* للمضادات الحياتية إن ***E.coli*** كانت مقاومه بنسبه (100%) للمضادات Amoxicillin,Ampicillin وTrimethoprime ولكنها حساسة بنسبة (100%) للمضادImipenem وتختلف نسبة مقاومة *E.coli* للمضادات الحياة الأخرى .لقد أظهرت بكتريا *E.coli* المنتجة (β- lactamases) واسعة الطيف مقاومة عالية لأنواع مختلفة من مضادات الحياة سميا الجيل الثالث من السفالوسبورين .

Introduction:

Extended spectrum beta lactamase (ESBLs) enzymes were first reported in Germany in 1983 from *Klebsiella pneumonia* and they are group of enzymes capable of hydrolyzing the third generation oxymino-cephalosporins such as (cefotaxime, ceftazidime, ceftriaxone), the monobactam (aztreonam) but not the cephamycin (cefoxitin,cefotetam) or carbapenems(imipenem,meropenem) [1,2,3,4]. ESBLs produced mostly by members of Enterobacteriaceae have emerged as serious nosocomial pathogens globally.[5,6] The persistent exposure of bacterial strains to ß-lactams induces mutation and continuous production of ß-lactamases in these bacteria, expanding their activity even against the third and fourth generation cephalosporins such as ceftazidime, cefotaxime and cefepime and against monobactams e.g. aztreonam. Thus these new ß-lactamases are called extended spectrum ß-lactamases (ESBLs), which are mostly plasmid mediated enzymes.[7] Although ESBLs have been reported more frequently from *Klebsiella pneumoniae* and *E. coli* but other members of Enterobacteriaceae and *Pseudomonas* spp. are also implicated for ESBL production.[7,8] .There are various reports of ESBL producing organisms worldwide from hospitals environments, patients, farm animals, sewages, food material.[9,10,11] Organisms producing ESBLs are clinically relevant and remain an important cause for failure of therapy with cephalosporins and other classes of antibiotics throughout the world1. Therefore it is necessary to know the

ESBL status of clinical isolates especially in tertiary care hospitals.

The spread of ESBLs in Gram-negative bacteria represents a major challenge to the antimicrobial therapy of infections caused by these organisms either in hospitals or in a community setting.[7]While definitive guidelines for the management of patients infected with ESBL-producing bacteria are still awaited, there is strong evidence that failure to detect ESBL-mediated resistance can lead to treatment failure.[12]. ESBLs have serine at their active sites which attack the amide bond in the lactam ring of antibiotics causing their hydrolysis. These enzymes which now number more than 150 were initially limited to Escherichia coli and *Klebsiella* species. Lately many have been spreading and are engulfing other genera specially *Enterobacter* and *Proteus*. ESBL phenotypes and detection have become more complex due to the diversity of the enzymes produced, emergence of inhibitor resistant ESBL variants plasmid borne resistance genes, Concurrent Amp-C production enzyme hyper production and porin loss. During the last decade a number of ESBL phenotype has been reported. The production of multiple enzymes, inhibitor resistant ESBL variant, emergence of CTX-M types of ESBLs, plasmid borne AmpC and production of ESBLs in AmpC producing strain, has rendered more complexity to the ESBL phenotypes.[13] During the late 1990s and early 2000s CTX-M producing enterobacteriacea has emerged as the most common ESBL type in many parts of the world including Africa, South America, Asia and some parts of Europe.[14,15]

In recent years there has been an increased incidence and prevalence of ESBLs, majority are derived from the widespread broad-spectrum ß-lactamases TEM-1, TEM-2 and SHV-1. There are also new families of ESBLs, including the CTX-M and OXA-type enzymes as well as novel, unrelated ß-lactamases.3 Several different methods like disk approximation or double disk synergy, modified double disc test (MDDT), NCCLs phenotypic confirmatory method, E-test ESBL strips, three dimensional test, Vitek system etc. have been suggested for the detection of ESBLs in clinical isolates.[16] While each of the tests has merit, none of the tests is able to detect all of the ESBLs encountered. Disk approximation or double disk synergy is one of the currently available and widely practiced techniques for the detection of ESBLs. Phenotypic tests (double-disk synergy test, ESBL E-test, and the combination disk method) are based on clavulanate inhibition and extended spectrum of cephalosporin (ESC) susceptibility testing. They often need slight changes by either reducing the distance between the disks of ESC and clavulanate.[17,18] Up till now, there is no gold standard method for ESBL detection but

NCCLS recommend the phenotypic method as confirmatory test.[19]

Materials and methods:

Methodology

Patients: The study included collected and examined 223 urine samples from suspected patients with urinary tract infections, admitted in Al- Sader medical city .

Culture and Antimicrobial susceptibility testing:

Following aseptic collection, urine samples were inoculated onto Blood agar and MacConkey agar media. The plates were incubated at 37°C aerobically and after overnight incubation, they were checked for bacterial growth. All organisms were identified by their colony morphology, staining characters, pigment production, motility and other relevant biochemical tests as per standard methods of identification[20]. All gram-negative bacteria isolates were tested for antimicrobial susceptibility by using commercially available antimicrobial discs on Mueller Hinton agar[19]. *E. coli,* were tested against ampicillin (30µg), cotrimoxazole (30µg), gentamicin ((30µg), ciprofloxacin (10µg), aztreonam (30µg), ceftriaxone(30µg), ceftazidime (30µg),amikacin(30µg),amoxicillin(25µg),and imipenem (30µg). Zone of inhibition was recorded as *Sensitive* or *Resistant* according to NCCLs chart.[21]

ESBLs detection

Modified double disc test (MDDT)[18]

Phenotypic determination of ESBL enzymes

ESBL detection was determined isolated bacteria namely *E. coli* using double disc synergy test. Briefly, a sterile Mueller-Hinton agar was prepared and a 0.5 MacFarland equivalent standard of the test organisms was streaked on the surface of the agar with a sterile loop and allowed for 15-20 mins to pre-diffuse. An Augmentin which is a combination of clavulanic acid 20 (µg) and amoxicillin (10 µg) was placed at the center of the petri-dish and cefotaxime (30 µg), ceftaxidime (30 µg), aztreonam (30 µg) ciprofloxacin (30 µg) were placed15mm apart center to centre on the plates with a sterile forceps. These were incubated at 35oC for 18-24 h. An enhanced zone of inhibition from 5 mm above in the presence of Augmentin is regarded as positive for phenotypic production of ESBL enzyme.

Phenotypic confirmatory test for ESBLs[19]

Confirmation of ESBL-producing isolates (MDDTpositive) was done by inhibitor potentiated disc diffusion test according to NCCLS recommendation.Combinations of ceftazidime and cefotaxime disc with lavulanic acid (10mg) were prepared an hour before their application to the Mueller Hinton plates inoculated with test bacteria (corresponding to 0.5 McFarland tube). Ceftazidime and cefotaxime

discs without clavulanic acid were placed on one side of inoculated plate and ceftazidime, cefotaxime discs combined with lavulanic acid were placed on other side of plate. Diameter of zone of inhibition was measured after overnight incubation at 37°C. A >5mm increase in a zone diameter for cefotaxime and ceftazidime tested in combination with clavulanic acid versus its zone when cefotaxime and ceftazidime were tested alone confirmed an ESBL producing organism.

Antibiotic susceptibility studies

Sensitivity of *E. coli* to different classes of antibiotics was performed by disc diffusion method. Briefly, a sterile Mueller-Hinton agar was prepared and a 0.5 MacFarland equivalent standard of the test organisms was streaked on the surface of the agar and allowed for 15-20 mins to pre-diffuse. The following antibiotics disc, ampicillin (30µg), cotrimoxazole (30µg), gentamicin ((30µg), ciprofloxacin (10µg), aztreonam (30µg), ceftriaxone (30µg), ceftazidime (30µg),amikacin(30µg),amoxicillin(25µg),and imipenem (30µg). were placed on the surface of the agar with a sterile forceps. These were incubated at 35oC for 18-24 h, after which the inhibition zone diameter in (mm) was taken.

Results:

A total of 235 urine samples were examine for presence of E. coli and those producing ESBL enzymes. E.coli was the most common organisms isolated from urine 104 (44.2).

Out of 78 *E. coli* isolated from urine(re tested for ESBL production and it was found that 30 (38.4) were MDDT positive and 27 phenotypic confirmatory test positive. Three strain of *E.coli* were MDDT. positive but negative by phenotypic confirmatory test. Table (2).

Antibiotic susceptibility studies of ESBL bacteria showed that *E.coli* isolated were totally(100%)resistant to ampicillin,amoxicillin and trimethoprim but the ESBL producing E.coli showed maximum susceptibility to imipenem(100 %) followed by amikacin(83.33%)and variably resistant to gentamicin(86.33%),ciprofloxacin(8 3.33%),ceftriaxone (80%),ceftazidem (70 %),and aztronam (60 %).

Table (I) Frequency of ESBL among *E.coli* that isolated from urine.

Organism	Urine n=235		Total.
E.coli(%)	Male	Female	
	29 (27.8)	75 (72.1)	104 (44.2)
No. of isolated tested	Male	Female	
	21	57	78
No. of ESBL producers (%)	Male	Female	
	8 (26.6)	22 (73.3)	30 (38.4)

Table (II). Comparative of modified double disk test (MDDT) and phenotypic confirmatory method for ESBL detection

Bacteria	MDDT positive	phenotypic confirmatory test positive.
*E.coli (*No. of ESBL producers= (30)	30	27

Figure1. MDDT of ESBL producing *E.coli.* Enhancement of zone of inhibition produced by susceptible strain of *E. coli* to 3rdgeneration cephalosporins and aztreonam to wards amoxyclav disc placed at the centre.

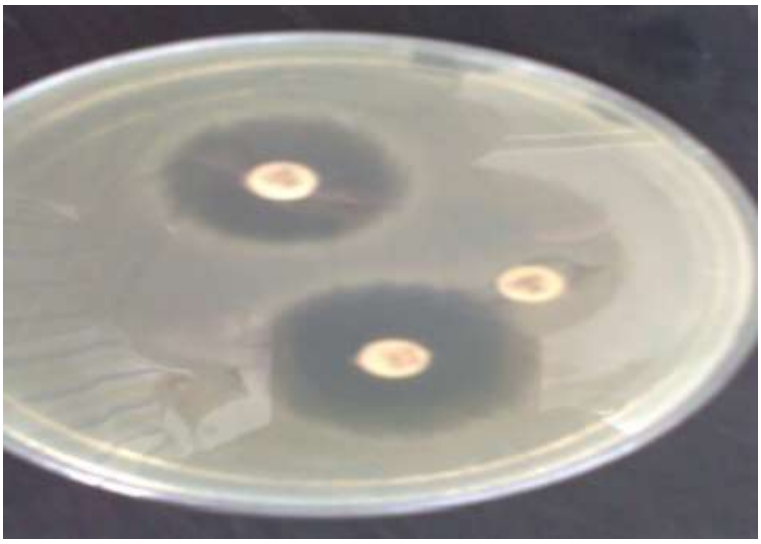

Figure-2. phenotypic confirmatory method for ESBL producing *E.coli*. Confirmed with ceftazidime-clavulanate (a) versus ceftazidime alone (b).

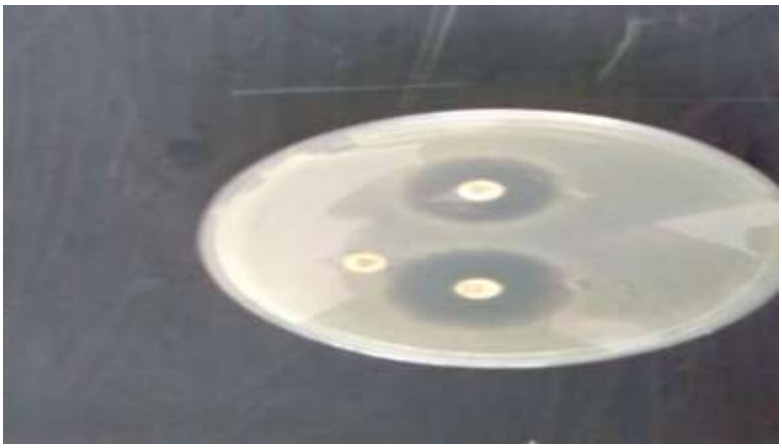

Figure-3. phenotypic confirmatory method for ESBL producing *E.coli*. Confirmed with cefotaxime -clavulanate (a) versus cefotaxime alone (b)

Table (3). Antibiotic resistant patterns of ESBL producing *E.coli.*

Antibiotics	*E.coli.*n=30 (%)		
	resistant	*intermittent*	*sensitive*
Ampicillin	30(100)	-	-
Amoxicillin	30(100)	-	-
Trimethoprim	27(90)	1(3.33)	2(6.66)
Gentamicin	26(86.33)	1(3.33)	3(10)
Amikacin	3(10)	2(6.66)	25(83.33)
Imipenem	0(0)	-	30(100)
Ciprofloxacin	25(83.33)	1(1.33)	4(13.33)
Ceftriaxon	24(80)	3(10)	3(10)
Ceftazidem	21(70)	2(6.66)	7(23.33)
Aztreonam	18(60)	2(6.66)	10(33.33)

Discussion:

In most instance, empirical antibiotics therapy of serious bacterial infection are one of the third cephalosporins, but in the last two decades, ESBL has emerged as a major contributor of cephalosporin resistant .spread of ESBLs in Gram-negative bacteria represent a major challenge to the antimicrobial therapy of infection caused by these microorganisms either in hospital or in community.[22]

the detection of ESBL-producing strains is very importance for all major hospitals worldwide, for a number of reasons, First, these strains are most likely to be even more prevalent than it is currently recognized. Due to the difficulty in their detection by the current clinical methods, many of these strains have been reported to be susceptible to widely used and tested broad-spectrum ß-lactams. Secondly, ESBLs constitute a serious threat to current ß-lactam therapy. Treatment of ESBL infection is difficult as the CLSI recommends that all expanded spectrum cephalosporins be taken resistant in ESBL producers. Thirdly, institutional outbreaks are increasing because of selective pressure due to the heavy use of expanded-spectrum cephalosporins and also due to lapses in effective infection control measures. [23,24]

Recent studies on ESBL production among the members of Enterobacteriaceae which were isolated from clinical specimens,showed an increase in the occurrence of ESBL producers.[7]A study from North India on uropathogens such as *Klebsiella pneumoniae*,*Escherichia coli*, *Enterobacter*, *Proteus* and *Citrobacter spp*.showed that 26.6% of the isolates were ESBL producers.[24]A study from Nagpur showed that 48.3% of their cefotaxime resistant gram negative bacilli were ESBL producers.[25]A

report from Coimbatore (India) showed that ESBL production was 41% in E. coli and 40% in K. pneumonia.[26] In the present study, we also observed that 30 (38.4%) of the E. coli isolates were ESBL producers. In similar study by Haque and Salam[27],43.9% of the *E.coli* isolates were reported to be ESBL producer, also Umadevi *et al*[28] showed that 47.83% 0f *E.coli* to be ESBL-producer, but Mathur *et al*[29]62% 0f the *E.coli* isolates were reported to be ESBL producer. However, ESBLs are wide spread the world over showed that, but the prevalence and phenotypic characteristics among clinical isolates may vary between geographic areas.[7]

In our study , antibiotics susceptibility test showed that ESBLs producing *E.coli* isolates were100%sensitive only to Imipenem ,while showed significantly increasing multi resistant to all other antibiotic used (table -3).rare use of Imipenem may be explain high susceptibility to this antibiotic . However, other studies of Subha *et al*[30]and Rodrigues *et al*[31]. showed ESBL-producing *E.coli* to be having the highest susceptibility to Meropenem (94.4%).

In conclusion, the ESBL *E. coli* are multidrug resistant so ,it may be represent a major problem in the area of infection disease. To help the physician in describe the proper antibiotic in such cases, it is essential to report ESBL production along with routine sensitivity reporting. The best antibiotics can be describe for ESBL bacteria are imipenem or meropenem.

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