Detection of *bla$_{SHV}$*, *bla$_{TEM}$* and *bla $_{CTX-M}$* among Urinary Tract Infection *Escherichia coli* isolates

Mun'im Radwan Ali **Maha Mekhlef Kahdim**
Department of biology -Collage of science- Al-Mustansiryah University.
Shahbaa Hamid Majeed
Departme of science -Collage of basic education- Al-Mustansiryah University.
mnumbio77@yahoo.com Maha1979.mk@gmail.com Shalubidi@yahoo.com

Abstract

One hundred fifty urine samples were collected from urinary tract infection patients. We obtained 40 isolates of *Escherichia coli* among these clinical samples. A proportion of UTI in females 93 (62%) is higher than that in males 57 (38%). All locally isolates are multi-resistant, the highest rate of resistance was seen with Amoxicillin/clavulanic acid, and Cloxacillin. The results showed high percentage 14(35%) of class C among locally isolates , while only 4(10%) are able to produce Class A ESβL , on the other hand (7/40) and (8/40) were ESβL neither Class A nor Class C Present and ESβL absent respectively , while among the 40 isolates only 7 isolates were Non-Typable. Three types of β-lactamases include ESβLs genes (*bla$_{SHV}$*, *bla$_{TEM}$*, *bla$_{CTX-M}$*) by the use of uniplex PCR assay. The *bla$_{SHV}$* was detected in 1 isolates (2.5%) of the UPEC isolates, while for the *bla$_{TEM}$* gene the result showed that in 27 detected isolates (67.5%), there is 23 isolates (57.5%) which indicates the prevalence of *bla$_{CTX-M}$* gene. The results showed the Co-existence of *bla$_{TEM}$*, *bla$_{CTX-M}$* in 30% of isolates, while one isolate *bla$_{SHV}$*, *bla$_{TEM}$*, *bla$_{CTX-M}$* in their genome component.

Keywords: *blaSHV*, *blaTEM* and *blaCTX-M*, PCR assay,Urinary tract infection, *E.coli*.

الخلاصة

جمعت 150 عينة ادرارمن مرضى مصابين بالتهاب المجاري البولية ،وقد اظهرت الدراسة وجود 40 عزلة تعود لبكتريا *Esherichia coli* من مجموع العينات الكلية .وقد وجدت ان حالات الاصابة بالتهاب المجاري البولية كانت عند النساء تفوق الرجال اذ بلغت لدى النساء(62%)93 بينما كانت نسبتهاعند الرجال(38%)57 ،اظهرت جميع العزلات البكتيرية المحلية قيد الدراسة ذات مقاومة عالية للمضادات الحيوية وكانت اعلى نسبة مقاومة لمضاد الاموكسلين ،حامض الكلافيولونك والكلوكساسلين،وقد بينت النتائج اعلى نسبة للمقاومة للعزلات المحلية التي تمتلك انزيمات واسعة الطيف صنف C وبنسبة (35%)14 بينما كانت (10%)4 منتجة لانزيمات الواسعة الطيف صنف A ، ومن جهة اخرى كانت (7/40) عزلة و(8/40) من العزلات غير منتجة لانزيمات واسعة الطيف لكل من صنف A و C على التوالي .وقد تم دراسة الجينات المشفرة لانزيمات واسعة الطيف bla_{SHV} ، bla_{TEM} ، bla_{CTX-M} بواسطة التفاعل التضاعفي لسلسلة الدنا ،وقد اظهرت النتائج ان عزلة واحدة وبنسبة (2.5%)كانت تملك جين blaSHV بينما كانت 27 عزلة وبنسبة (67.5%) تملك جين blaTEM و23 عزلة بنسبة (57.5) تحمل جين bla CTX-M واظهرت النتائج ان 30 % من العزلات كانت منتجة لكل من انزيمات bla TEM و bla CTX-M بينما فقط عزلة واحدة كانت تملك 3 جينات مشفرة وهي blaSHV و bla TEM و bla CTX-M.

الكلمات المفتاحية:اشريشيا كولاي، التهاب المجاري البولية، PCR assay ,blaSHV , blaTEM and blaCTX-M.

Introduction

The β-lactams, such as penicillins, cephalosporins and carbapenems are the most commonly used antibiotics for treatment of nosocomial infections (Wright, 2000). Resistance to broad spectrum beta-lactams, mediated by extended spectrum beta-lactamase (ESBL), AmpC beta-lactamases (AmpC), and metallo-beta-lactamase (MBL) enzymes are an increasing problem worldwide , Presence of the latter two enzymes in clinical infections can result in treatment failure if one of the second- or third-generation cephalosporins is used (Singhal *et al*.,2005). Extended-spectrum _-lactamases (ESBLs)

were first described in the 1980s and they have been detected in *Klebsiella* species, and later in *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens* and other gram-negative bacilli (Kiratin *et al*., 2008). During the last century, bacteria has evolved the mechanisms to resist against antibiotics. Resistance to β-lactam antibiotics may be occurred as a result of permeability barriers, efflux pumps**,** altered penicillin binding proteins, AmpC-type β- lactamases and other products of β-lactamases (Conceição *et al*., 2005). Release of β-lactams, gram negative bacilli that harbored mutated versions of the potent TEM and SHV enzymes were detected. These and other newly detected β-lactamases (for example CTX-M) hydrolyze β-lactam antibiotics containing the oxymino side-chain (Paterson *et al*., 2004). CTX-M preferentially hydrolyze cefotaxime and based on the changes in amino acids sequences identities is divided into five groups. In the present study, we detect ESBLs among the *E. coli* isolates cultured from UTI-patients. Morever, the determination of susceptibility patterns against third-generation cephalosporins and identify the types of extendedspectrum beta-lactamases (ESBL) blaSHV, blaTEM and bla CTX-M, beta-lactamase genes in ESBL producing isolates were also targeted. In an attempt to make detection of these markers of drug resistance part of the daily activities of a diagnostic microbiology laboratory, to reduce the suffering of patients as a result of taking the treatments are inappropriate

Material and Methods

Collection of Urine Samples: One handrad fifty urine samples were collected during June to November 2011 from (1 year to 73 years) for isolation of *E. coli*. A total of 40 isolates of *E. coli* were studied and taken from UTI patients who were studied clinically suspected to suffer from urinary tract infection according to (Chessbrough ,1993)

Isolation and Identification of *Escherichia coli*: For isolation of bacteria urine samples were first inoculated into MacConkey agar and incubated at 37°C for 24 h, after which a loopful was spread on to plates of Eosin Methylene Blue and further incubated at 37°C for 24 h. Isolates were further purified by picking discrete colonies (green metallic sheen) and sub culturing onto fresh plates of EMB and further incubating for 18 to 24 h at 37°C .After incubation, 1 to 2 discrete colonies were inoculated into the presumptive diagnostic medium sulfide indole motility medium (SIM) and incubated at 35°C for 24 h. Further characterization of isolates was carried out using the IMVIC test. Isolates that were indole positive ,hydrogen sulfide negative, non-motile, as well as methyl red, Vogues – Proskauer and citrate utilization tests were identified as *E. coli*, fermentation of lactose, ability to produce indole, reaction on triple sugar iron (TSI) medium, hemolysis on blood agar, citrate utilization and motility of organism (Atlas *et al*., 1995).The identifications were confirmed by the API 20E test system (Bio-Merieux).

Detection of Susceptibility to Antibacterial Agents: Susceptibility of all the isolates to different antibiotics was determined by the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI). The antibiotic discs used in this study were Amoxicillin/clavulanic acid, Cloxacillin , Cefotaxim, CoTrimoxizole (Trimethoprim-Sulfamethaxazole) , Naldixic acid , Nitrofurantoin,

Ciprofloxacin, Ofloxacin, Norfloxacin and Pifloxacin .Each antibiotic concentration was applied on the surface of Muller -Hinton agar plates inoculated with E. coli isolates and incubated at 37°C for 24 h. (Tankhiwale *et al*., 2004).

Detection of the expression of AmpC gene and ESβLs: In order to simultaneously detect ESβL and AmpC, a modified double disk approximation method (MDDM), was devised. A 0.5 McFarland of test isolate was swabbed on Mueller Hinton Agar (Difco) plates and disk of Cefotaxime (30 µg) and Ceftazidime (30 µg) were placed adjacent to Clavulanic acid (10 µg) and Cefoxitin (30 µg) disk at a distance of 20 mm from each other. After incubation, an enhanced zone of inhibition between any of the disk Ceftazidime and Clavulanic acid were interpreted as presumptive evidence for the presence of ESβL. Isolates showing blunting of Ceftazidime zone of inhibition adjacent to Cefoxitin disk or showing reduced susceptibility to either of the above test drugs (Ceftazidime) and Cefoxitin were considered as "screen positive" and were selected for detection of AmpC β-lactamases. (20). The following classification was used. (Sinha *et al*., 2008)

- Class A ESβL present:
  (i) Potentiation of the inhibition zone (IZ) of any one of Cefpodoxime, Ceftazidime, Ceftriaxone, or Aztreonam when combined with Clavulanic acid.
  (ii) Susceptibility (S) to Cefoxitin.
  (iii) S or resistance (R) to any one of Ceftazidime, Ceftriaxone, or Aztreonam.
- Class A and Class C ESβL present:

(i) Potentiation of the IZ of any one of Cefpodoxime,Ceftazidime, Ceftriaxone, or Aztreonam when combined with Clavulanic acid.
(ii) R or intermediate (I) to cefoxitin.
(iii) S or R to any one of Ceftazidime, Ceftriaxone, or Aztreonam.

- Class C ESβL present:
  (i) No potentiation with Clavulanic acid.
  (ii) R or I to Cefoxitin.
  (iii) R to any one of Ceftazidime, Ceftriaxone, or Aztreonam.
- ESβL not Class A or Class C present:
  (i) No potentiation with Clavulanic acid.
  (ii) S to Cefoxitin.
  (iii) R to any one of Ceftazidime, Ceftriaxone, or Aztreonam.
- ESβL absent
  (i) No potentiation with Clavulanic acid.
  (ii) S or R to Cefoxitin.
  (iii) S to all of Ceftazidime, Ceftriaxone, or Aztreonam.All the isolates were simultaneously subjected to AmpC disc test for AmpC βL detection

Preparation of bacterial DNA: The DNA to be amplified was extracted from whole organisms by boiling method. The bacteria were harvested from 1.5 ml of an overnight Luria-Bertani broth culture, suspended in sterile distilled water, and incubated at 95 C◦ for 10 min. Following centrifugation of the lysate, the supernatant was stored at -20 C◦ as a template DNA stock (Farshad *et al* ., 2010) .

Uniplex PCR amplification procedure: Three primer sets were used to amplify an internal region of the *blaTEM, blaSHV and bla CTX-M* genes according to (Kalantar *et al*., 2010) . The primers sequences were previously reported by and obtained from Alpha DNA company (USA). Amplification was performed in a thermal

cycler (Eppendorf, Germany). Using the following primers for *blaTEM* F(**5′**-GAGTATTCAACATTTCCGTGTC-**3′**).*blaTEM* R (**5′**-TAATCAGAGGCACCTATCTC-**3′**); the reactions mixtures included an initial denaturation at 94°C for 5 min consisted of 35 cycles of 94 ºC for 30 seconds , specific annealing temperature 48 C◦ for 60 seconds and 72 ºC for 5 min 30 seconds, and a final extension at 72 ºC for 10 min. while *blaSHV* F(**5′**- AAGATCCACTATCGCCAGCAG-**3′**), *blaSHV* R(**5′**- ATTCAGTTCCGTTTCCCAGCGG - **3′**); the reactions mixtures included an initial denaturation at 94°C for 5 min consisted of 35 cycles of 94 ºC for 60 seconds , specific annealing temperature 59C◦ for 60 seconds and 72 ºC for 60 seconds, and a final extension at 72 ºC for 10 min. for *CTX-M F(5′- CGCTTTGCGATGTGAAG-3′), CTX-M R(5′- ACCGCGATATCGTTGGT - 3′)* gene the reactions mixtures included an initial denaturation at 94°C for 5 min consisted of 35 cycles of 94 ºC for 60 seconds , specific annealing temperature 55C◦ for 60 seconds and 72 ºC for 60 seconds and a final extension at 72 ºC for 10 min in Thermal Cycler. The detection PCR products was performed on 0.8 to 1% agarose gels by electrophoresis and visualized under UV light.

Results and Discusion

Isolation of *Escherichia coli*

One hundred fifty urine samples were collected from urinary tract infection patients. We obtained 40 isolates of *Escherichia coli* among these clinical samples. These were compatible with Abass *et al* (2014) who found that *E.coli* is the major causative agent in human UTIs, one of the most common bacterial infections. Virtually any bacteria can occasionally cause an UTI but irrespective of sex, age and geography *E. coli* is the dominating pathogen (Naber.,2008) and is the causative agent in 90% of the cases in younger patients with uncomplicated UTI (Kahlmeter.,2003). In older women the etiological spectrum is more complex (Molander *et al*.,2000) .

Table 3-1: Demographic data of *Escherichia coli* UTI patients

Variable	Data
Age of patients	1year -73 years
Sex of patients Female : male	 93:57
Clinical case in adult women UTI Clinical case in girls UTI Clinical case in men UTI Clinical case in children UTI	70 cases 22 cases 46 cases 12 cases

This table indicates that, a higher proportion of UTI in females 93 (62%) than that in males 57 (38%). This is understandable due to the anatomy and is a consistent trend worldwide .The study is in agreement with (Jadhav *et al*., 2011).

UTIs are common infections and the increased antimicrobial resistance hinder their treatment. The most commonly used antibacterial drugs in the treatment of UTI are trimethoprim-sulfamethoxazole, cephalosporins, semi-synthetic penicillins with or without beta-lactamase inhibitors and quinolones (Chung *et al*., 2010).

It has been observed that all isolates are multi-resistant , the highest rate of resistance is seen with Amoxicillin/clavulanic acid, and Cloxacillin and are moderately

resistant to Cefotaxim, CoTrimoxizole (Trimethoprim-Sulfamethaxazole) and Naldixic acid whereas some isolates have shown lowest rates of resistance to Nitrofurantoin, Ciprofloxacin, Ofloxacin, Norfloxacin and Pifloxacin .

Phenotypic methods are not able to distinguish between the specific enzymes responsible for ESβL production (SHV, TEM, and CTX-M types). Several research or reference laboratories use genotypic methods for the identification of the specific gene responsible for the production of the ESβL, which have the additional ability to detect low-level resistance (i.e, can be missed by phenotypic methods). Furthermore, molecular assays also have the potential to be done directly on clinical specimens without culturing the bacteria, with subsequent reduction of detection time (Farkosh, 2007).Unfortunately, extensive use of antibiotics is the cause of resistance phenomena, and treatment of these infections especially nosocomial infections faces a serious problem. ESβLs are enzymes that confer resistance to third generation cephalosporins and monolactams. A large number of plasmid-transferable ESβLs capable of inactivating extended-spectrum cephalosporins have been discovered (Bradford, 2001).

The results showed (Table 2) high percentage 14(35%) of class C among locally isolates, while only 4(10%) are able to produce Class A ESβL , on the other hand (7/40) and (8/40) were ESβL niether Class A nor Class C Present and ESβL is absent respectively , while among 40 isolates only 7 isolates were Non-Typable.

Table 2: Number and Percentage of extended spectrum beta lactamase producers as per classification mentioned

Class A ESβL Present	Class A & Class C ESβL Present	Class C ESβL Present	ESβL not Class A or Class C Present	ESβL Absent	Non-Typable
4(10%)	-	14(35%)	7(17.5%)	8(20%)	7(17.5%)

In this study, total of 40 UPEC isolates were recovered during the study period to use for detecting three types of β-lactamases including ESβLs genes (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$) by the use of uniplex PCR assay. The genotyping results of ESβLs producing isolates obtained by uniplex PCR amplification of SHV, CTX-M,TEM is shown in table (2).

Table 3:The β-lacatamses genes and Percentage pattern of *E.coli* from pregnant woman:

ESβLs β-lacatamses genes	Number of positive isolates (N40)	Percentage of positive isolates (N40)
SHV	1	2.5%
CTX-M	23	57.5%
TEM	27	67.5%
CTX-M &TEM	12	30%
SHV, CTX-M &TEM	1	2.5%

In the current study the result showed that the *bla*$_{SHV}$ was detected in 1 isolates (2.5%) of the UPEC isolates using boiling method. The result of *bla*$_{SHV}$ gene is not identical with Pakzad *et al.*(2011) where the findings revealed the emergence of a high proportion of the *bla*$_{SHV}$ gene that was 95.2% of *E.coli* isolates. while Pongpech *et al.* (2008) reported that the frequency of *bla*$_{SHV}$ gene was 8% of the confirmed ESBL producing *E. coli* isolates

While for the *bla*$_{TEM}$ gene the result showed that it was detected in 27 isolates (67.5%) of the UPEC isolates. This result of the current study is in line with the result of Almohana (2013) that showed that the *bla*$_{TEM}$ gene which was (57.1%) of *E.coli* isolates. The variation in our study results compared with others about prevalence rate of *bla*$_{SHV}$ and *bla*$_{TEM}$ may be raised from different reasons such as the difference in type and volume of consumption of antibiotics and difference in time during which the isolates were collected (Al-Agamy *et al*., 2009).

The current results showed the prevalence of *bla*$_{CTX-M}$ gene (amplified size 550bp) also which was chosen for the genotypic detection for ESβL was also present in the 23 isolates (57.5%). The results of *bla*$_{CTX-M}$ gene of the current study which are similar with the results of Chaudhuri *et al*. (2011) also showed a total of 63.5% of ESBL producers isolates carried *bla*$_{CTX-M}$. The *bla*$_{CTX-M}$ has detected the most common type of *bla*$_{CTX-M}$ from Asia, Europe , North and South America among multidrug-resistant *E .coli* (Feizabadi *et al* ., 2010).

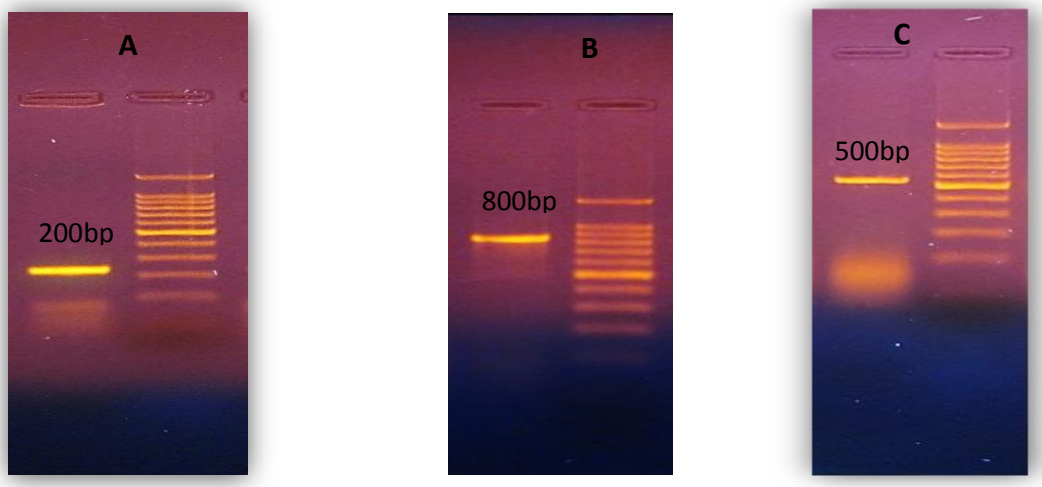

Figure1: Agaros gel electrophoresis(1% agarose, 7 v/cm^2) and Ethidium bromide staining to detect A: *bla*$_{SHV}$ gene size product (band 200bp); B: to detect *bla*$_{TEM}$ gene size product (band 800bp); C: to detect *bla*$_{CTX-M}$ gene size product (band 550bp) by using template DNA prepared by boiling method. Right Lane , molecular size DNA ladder (100 bp DNA Ladder); Left lanes DNAs isolated from *E. coli* sample showed Positive PCR bands.

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