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Molecular Detection of *AmpC* Family Genes Encoding Antibiotic Resistance among *Escherichia coli* isolated from Patients with Urinary Tract Infection (UTI) in Najaf Hospitals

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Abstract :

One hundred and thirty significant bacteriuria were detected. The study showed higher incidence of UTI in females (73.08%) than males (26.9%). The *E. coli* isolates which grown on culture of all the 130 urine samples with significant bacteriauria were 60 (46.2 %) . The isolates which were resistant to ampicillin and amoxicillin 53(88.3%) . The ability of *E. coli* isolates to *AmpC* production were tested; the results have revealed that 23 (43.4%) isolates produce *AmpC* β-lactamase. PCR amplification results have shown that eighteen *E. coli* isolates possess *AmpC* β- lactamase gene. The results in this study showed 8 (44.4%) had bla_{FOX} gene and 7(38.9%) had bla_{CIT} while 5(27.8%) of *E. coli* isolates had bla_{DHA} and 9(50%) had bla_{EBC} gene.

Key words: Detection AmpC β-lactamaseEscherichia coli, Prevalence bla_{EBC}gene , Urinary tract infection

التشخيص الجزئي للجينات المقاومة Ampcالمشفرة للمقاومة للمضادات في عزلات بكتريا القولون المعزولة في التهاب المجاري البولية

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الخلاصة:

أظهرت النتائج في هذه الدراسة أن 130من عينات الإدرار المأخوذة من المرضى المشكوك بإصابتهم بالتهاب المسالك البولية كانوا مصابين فعلا بالبيلا البكتيرية significant bacteriuria. كما انه عدد عزلات الاشريشيا القولونية التي تم الحصول عليها من 130عينة إدرار كانت 60عزلة وكانت هذه العزلات قد خضعت لاختبار الحساسية لكل من المضادين الحيويين هما الامبيسيلين والاموكسيسلين وأظهرت النتائج أن 53 منها كانت مقاومة لكلا المضادين . وعندما اختبرت هذه العزلات بواسطة اختبار ثلاثي الأبعاد واختبار الأقراص لمعرفة قدرتها على إنتاج أنزيم ألبيتا لاكتاميز أظهرت النتيجة ان 23عزلة منها أنتجت هذا الإنزيم ولكن عند استخدام تقنية PCRأظهرت النتيجة أن 18عزلة منها كانت حاملة جين AmpCثم بعد ذالك خضعت لاختبار أربعة جينات من عائلة AmpC وهي bla_{FOX} و bla_{CIT} وكانت نسبها هي 8(44.4%) و 7(38.9%) على التوالي . والجينيين الآخرين هما bla_{DHA} و bla_{EBC} وكانت نسبهما 5(27.8%) و9(50%) على التتالي .

Introduction:

Urinary tract infection (UTI) is remains one of the most common bacterial infections and second most common infectious disease in the community practice. Approximately about 150 million people were diagnosed with UTI each year (1) .Uropathogenic *E. coli* cause 90% of the UTI in anatomically normal, unobstructed urinary tracts.

The bacteria colonize from the feces or perineal region and ascend the urinary tract to the bladder the typical patient with uncomplicated cystitis is a sexually active female who was first colonized in the intestine with UPEC strain.

The organisms are propelled into the bladder from the per urethral region during sexual intercourse, with specific adhesions they are able to colonize the bladder (2). The bladder infection are 14- times more common in females than male which attributed to anatomical differences between the genders women have shorter, less complex urethras than men, and the urethral opening is much closer to the anus, facilitating transfer of uropathogens from the gastrointestinal tract into the urinary tract also absence prostate fluide which has antibacterial characteristics (3).

One of the most important mechanisms of antibiotic resistance in Uropathogenic *E. coli* is the production of *AmpC* β-lactamases (Class C β-lactamases) which are an important group of enzymes that are broadly distributed in the world; it is the second most common β-lactamase group (4). The first bacterial enzyme reported to destroy penicillin was the AmpC β-lactamase of *E. coli*, although it had not been so named in 1940 (5). The inducible chromosomal *AmpC* genes were detected on plasmids of *Klebsiella* spp., *E. coli*, or *Salmonella* spp. In *E. coli AmpC* is poorly expressed, while in *Klebsiellae* and *Salmonella* species the *AmpC* gene is missing from the chromosome and found on the plasmids (6). Recently, more than 100 different *AmpC* enzymes were commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria (7).

Methods:

Collection of specimens

The present study included collection of 250 urine samples from three hospitals in Najaf (Al-Sader Medical City , Al-Hakeem General Hospital, Al-Furat Teaching Hospital) during the period from (January 2012 to April 2012). Out of the 250 samples 178 were female and 72 were male. The samples were taken by standard mid-stream "clean catch" method from patients with suspected urinary tract infections and each urine sample was collected from patient into a sterile container (8,9). The specimens were transferred immediately to the laboratory for culture and identification.

Isolation and Identification of Bacterial Isolates

The urine sample recovered from patients suspected with bacteruria and divided into two portions. One portion was for the direct microscopic examination, the urine samples were mixed and aliquots centrifuged at 5000 rpm for 5 min. The deposits were examined using both x10 and x40 objectives. Samples with ≥10 white blood cells/mm^3 were regarded as pyuric (10). A volume of the urine samples were applied to a glass microscope slide, allowed to air dry, stained with gram stain, and examined microscopically (11) .The second was cultured firstly on brain heart infusion broth and then sub-cultured on MacConkey agar and Eosin methylene blue agar using standard loop method.

The MacConkey agar is specially made to distinguish lactose fermenting (pink to red colonies) from non lactose fermenting bacteria (colorless or slightly beige)and incubated for overnight at 37°C.The culture results were interpreted as being significant and insignificant bacteriuria, according to the standard bacteriological

tests. A growth of $\geq 10^5$ colony forming units/ml was considered as significant bacteriuria (12,13).The pure cultures were prepared for biochemical tests to differentiate *E. coli* from other *Enterobacteriaceae* depending on biochemical tests (positive for methyl red and indole tests, negative in the Voges-Proskauer, Simmon citrate, and urease tests), acid/acid with gas production in the triple sugar iron test (14).

Detection of *AmpC* Beta-lactamase

a) Modified Three Dimensional Test (MTDT)

This test was carried out according to Manchanda and Singh (15) and Parveen (16)as follows:

Fresh overnight growth from Muller-Hinton agar plate was transferred to a pre-weighed sterile Eppendorf tube. The tube was weighed again to ascertain the weight of the bacterial mass. The technique was standardized so as to obtain 15 mg of bacterial wet weight for each sample. The growth was suspended in peptone water and pelleted by centrifugation at 3000 rpm for 15 min. Crude enzyme extract was prepared by repeated freeze-thawing approximately 15 cycles. Lawn cultures of *E. coli* ATCC 25922 were prepared on Muller-Hinton agar plates and cefoxitin 30µg disks were placed on the plate. Linear slits 3 cm were cut using asterile surgical blade 3 mm away from the cefoxitin disk. Small circular wells were made on the slits at 5 mm distance, inside the outer edge of the slit by stabbing with a sterile pasteur pipette on the agar surface.

The wells could easily be loaded with the enzyme extract 30µl increments until the well was filled to the top. the plates were kept upright for 5-10 min until the solution dried, and then incubated at 37^{o}C overnight. The isolates showing clear distortion of the zone of inhibition of cefoxitin were taken as AmpC producers. The isolates with no distortion were taken as AmpC non producers and isolates showing minimal distortion were taken as indeterminate isolates.

b) AmpC Disk Test

All isolates subjected to MTDT were also simultaneously checked byAmpC disk test. A lawn culture of *E. coli* ATCC 25922 was prepared on Muller-Hinton agar plate. Sterile disks 6 mm were moistened with sterile saline 20µl and inoculated with several colonies of test organism. The inoculated disk was then placed beside a cefoxitin disk almost touching on the inoculated plate. The plates were incubated overnight at 37C apositive test appeared as flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test had an undistorted zone (16,17,18).

Molecular Detection of *AmpC*

Genomic DNA Mini kit

Bacterial DNA was extracted by using the Genomic DNA Mini kit(Geneaid) according to the manufacturer's instructions.

Mutiplex PCR Mixture

All *bla*$_{\text{AmpC}}$ gene positive isolates were subjected to four genes family of plasmid mediated AmpC β-lactamase (PABL),by using lyophilized AccuPower® PCR PreMix multiplex PCR protocol. Single reaction (final reaction volume 20 µl) consisted of plasmid mediated AmpC β- lactamase genes primers10 pmole/µl (consist of forward 1.5 µl and reverse 1.5 µl for each primer) , DNA template5µl were transferred to AccuPower® PCR tube and the reaction volume completed with 9µl nuclease free water to 20µl. The lyophilized blue pellet was dissolved by vortexing and briefly spin down. All materials were mixed in same AccuPower® PCR tube on ice bag under sterile condition.

PCR Thermocycling Conditions

Table (2-1): Programs of PCR thermocycling conditions

Multiplex gene	Temperature (^{o}C)/ Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
blaFOX	94/3min	94/30 sec	64/30 sec	72/1min	72/7min	25
blaCIT	95/2min	95/30sec	61/30sec	72/50sec	72/5min	25
blaEBC	95/2min	95/30 sec	62.6/30sec	72/40sec	72/5min	25
blaDHA	95/2min	95/30sec	61/30 sec	72/50sec	72/5min	25

PCR products were resolved on 1% agarose gels stained with ethidium bromide, and photographed with UV illumination.

Results and Discussion:

Specimens Collection and Identification

The present study included a collection of 250 urine samples from three hospitals in Najaf during the period from(January 2012 toApril 2012). out of the 250 samples processed ; 130 (52 %) showed significant bacteriuria (table 3-1) ; and 100 (76.9 %) of which showed significant pyuria (when more than 10 plymorphonuclear pus cells/high power field). Atotal of 95 (73.08%) females and 35(26.9%) males had positive urine culture with significant bacteriuria .

Type of culture	No.	Percentage	Female	Male
Significant bacteriuria	130	52%	95 (73.08%)	35 (26.9%)
Non-significant bacteriuria	23	9.2%	12 (52.2%)	11 (47.8%)
Sterile	97	38.8%	71 (73.2%)	26 (26.8%)
Total	250	100%	178 (71.2%)	72 (28.8%)

Table (3-1): Incidence of significant bacteriuria in patients suspected UT

However, the bacterial isolates that obtained as a pure and predominant in growth in urine samples were considered in the present study, all these bacteria were identified based on colonial morphology, and biochemical reactions according to (14,19,20). In the present study, the organisms which grown on culture of all the 130 urine samples with significant bacteriauria were as follows: 60 (46.2 %) isolates of *E. coli* and 70 (53.8%) were other gram negative and positive bacteria (table 3-2). The *E. coli* isolates were most common pathogens isolated from patients with significant bacteriuria in the group studied .

Bacterial isolate	No. of isolates		Total
	Female	Male	
E. coli	46(76.7%)	14 (23.3%)	60 (46.2%)
other Gram(-) and(+) isolates	54 (77.1%)	16 (22.9%)	70 (53.8%)
Total	100(76.9%)	30(23.1%)	130(100%)

Table (3-2): Distribution of etiological agents in patients with significant Bacteriuria .

Detection of *AmpC* β-lactamases-Producing Isolates

In the present study, an attempt was made to evaluate the frequency of β-lactam resistant in all *E. coli* (n=60) isolates which obtained from urine of patients with significant bacteriuria. The isolates were screened on Muller-Hinton agar supplemented with ampicillin and amoxicillin (each alone) .Such two β-lactam antibiotics were selected because they are the most commonly used antibiotics in the therapy of bacterial infections, compared to other β-lactam antibiotics. A part from their therapeutic usage, these antibiotics can provide a comprehensive primary screening of β-lactam resistant isolates because the isolate that is resistant to carbenicillin and cephalosporin is already resistant to ampicillin and amoxicillin(4,2). The results obtained in this study revealed that 53 (88.3%) of *E. coli* isolates were resistant to both ampicillin and amoxicillin. The results revealed that 23 (43.4%) of *E. coli* isolates producing *AmpC* from the total number of 53 *E. coli* isolates which were isolated from urinary tract infections (Figure 1). The results indicated that all 23 isolates have plasmid originated *AmpC* (Figure 2).

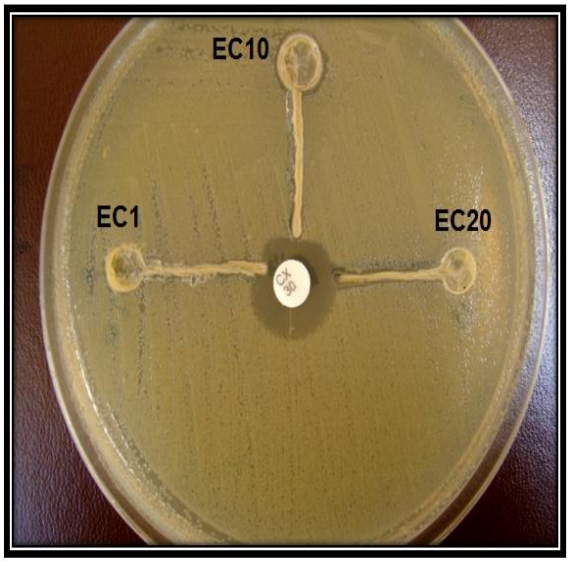
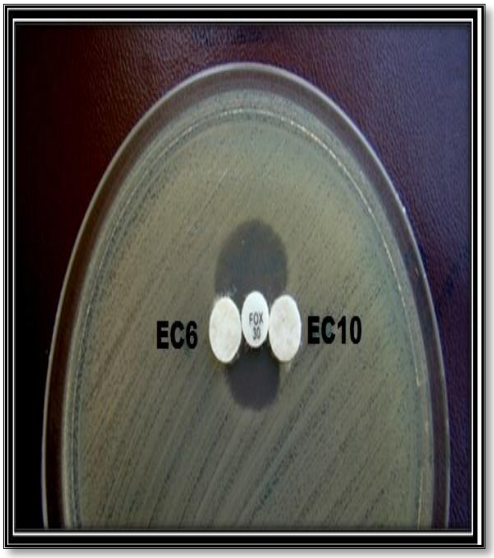


Figure (3-1): A-Three dimensional test, EC10 and EC1gave positive results and EC20 isolates gave negative results in this test.



Figure(3-2): B- AmpC disc test , EC6 and EC10 gave positive results in this test.

These findings are in agreement with those of Tenja (22). who found that the chromosomally encoded *AmpC* β-lactamases are very rare. Another study showed the susceptibility to third generation cephalosporins (e.g. ceftazidime and cefixime) serve as a poor marker for the identification of *AmpC*-producing bacteria (23). As well as the plasmid mediated *AmpC*- producing isolates can some times appear falsely susceptible to the third generation cephalosporins(24). The bacterial isolates producing PABLs have been found to be now wide spread for example, In the United States, occurrence rates of PABLs for *E. coli* were (4%)(25), In switzerland , (0.2%) of PABLs for *E. coli* [26], In China, found that only (2.0%) *E. coli* isolates were detected as PABLs (27) .

Molecular Detection of *AmpC* β-lactamase

Polymerase chain reaction technique has been used to amplify genes which encoding the *AmpC* β-lactamase. The results clarify that 18 isolates carrying *AmpC* gene (Figure 3-3and figure 3-4).

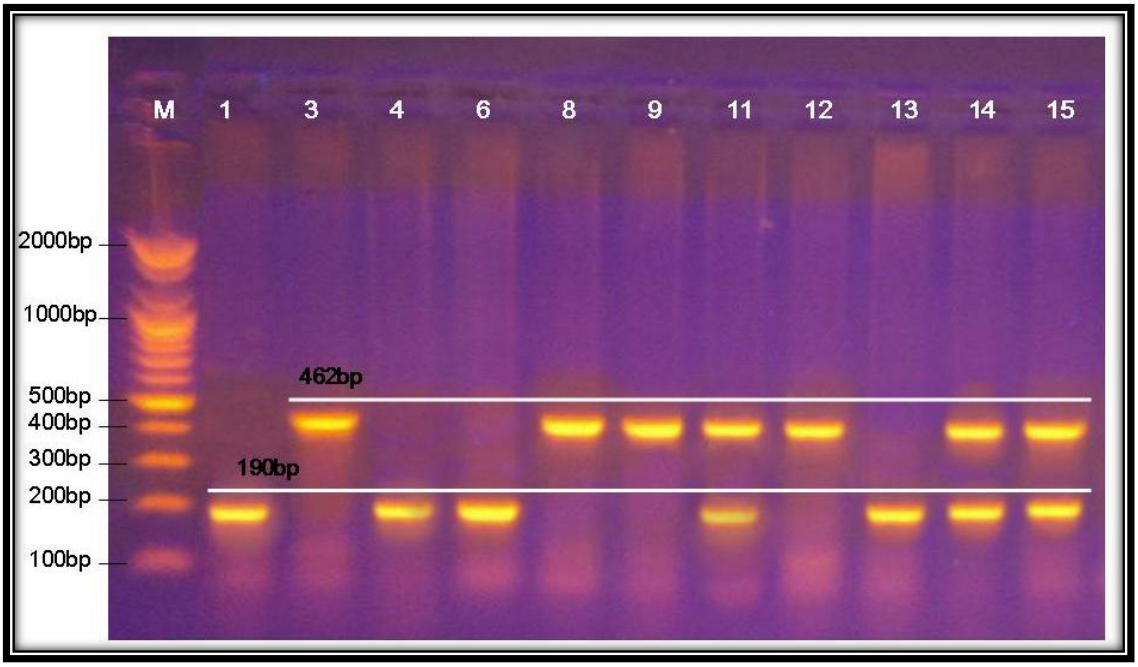


Figure (3-3): Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted DNA of *bla*AmpC positive *E. coli* isolates and amplified with bla_{CIT} and bla_{FOX} genes primers (forward and reverse). Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (EC3, 8, 9,11 , 12 ,14 and 15) of *E. coli* isolates showed positive results with *bla*CIT (462 bp) genes and Lanes (EC1, 4, 6, 11,13, 14,and 15) showed positive results with *bla*FOX (190 bp) genes .

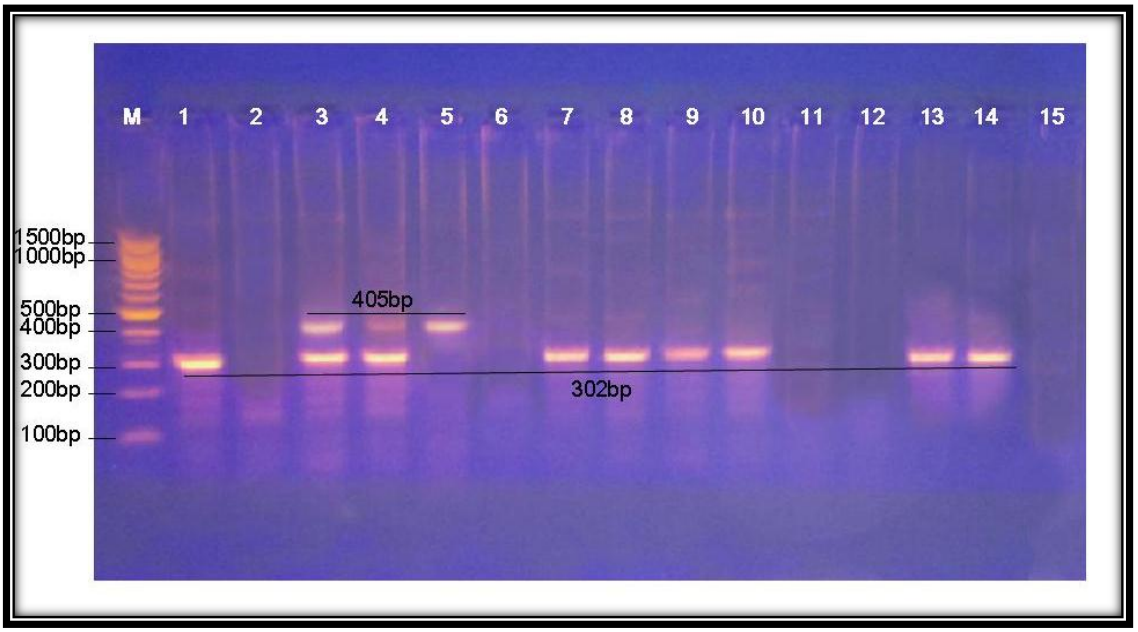


Figure (3-4): Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted DNA of *bla*AmpC positive *E. coli* isolates and amplified with bla_{DHA} and bla_{EBC} genes primers (forward and reverse). Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (EC3, 4 and 5) of *E. coli* isolates showed positive results with *bla*DHA (405 bp) genes and Lanes (EC1, 3, 4, 7,8, 9,10 ,13 and 14) showed positive results with *bla*EBC (302 bp) genes .

The results in this study showed 8 (44.4%) had a bands compatible with *bla*FOX gene and this result was higher than the results which reported in pakistan 2/121 (1.7%) *E. coli* isolates showing FOX *ampC* β-lactamase (28). Another study in Korea, *E. coli* resistance due to FOX enzyme have increasingly been noted, and a study performed in 2003 showed that high portion (53.4 %) of FOX resistance in *E. coli* was due to plasmid-borne AmpC *β*-lactamase production (29). wheares the CIT family (CMY2-7, LAT14 and BIL-1) was presented in (38.9%) of *bla*AmpC positive *E. coli* isolates. This prevalent rate was agreement with those reported by Tan *et al* who found that among 174 isolates of AmpC positive *E. coli* , this bacteria was most microorganisim expressed CIT enzyme 67 (39 %) (30) .

This investigation revealed that among the AmpC β-lactamase producers, 5 (27.8%) showed the presence of bla (DHA-1 and -2). This represents the first report of *bla*DHA in the Najaf. In accordance with Mohamudha *et al* who observed that DHA group genes were predominantly in *E. coli* 24/60 (40%) followed by in *K. pneumoniae* 19 (44.1%) (31).

Additionally, a new plasmid-mediated AmpC β-lactamase, which
was designated EBC (ACT-1 and MIR-1) family, was found in Najaf hospitals. Among the 18 *E. coli* isolates with blaAmpC gene, 9 (50%) had *bla*EBC type gene . In comparison with other studies, reported that EBC enzyme was the most common gene (21/57, 36.3%) among plasmid mediated β-lactamases producing enteric bacteria (32) .

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