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 blaFOX gene and 7(38.9%) had blaCIT while 5(27.8%) of E. coli isolates had blaDHA and 9(50%) had blaEBC gene.

Key words: Detection AmpC β-lactamase Escherichia coli, Prevalence blaEBC gene, Urinary tract infection

التشخيص الجزئي للمقاومة للمضادات في عزلات بكتريا القولون
المتميزة في التهاب المجاري البولية

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الخلاصة:
أظهرت النتائج في هذه الدراسة أن 130 من عينات الإدرار المأخوذة من المرضى المعالجين بمضادات تأثيرها المكمل الممارس للأمراض التي تسببها البكتيريا Escherichia coli البولية كانوا مضادين علاجًا بالبلاسمورين القولونوية التي تم الحصول عليها من 130 عينة إدرار كانت 60 عزلة وكانت هذه العزلات قد خضعوا لاختبارات الكلاسيكية لكل من المضادات الحيوينية والأمسيليسيلين وأظهرت النتائج أن 53 منها كانت مقاومة لكل المضادات. وعندما اختبرت هذه العزلات بواسطة اختبار ثلاثي الأبعاد، واختبار الأقرارات لمنع انتقال البكتيريا على إنزيمات ألكاميس، أظهرت النتيجة أن 23 عزلة منها انتهت هذا الإيزيم ولكن عند استخدام تقنية PCR عزلة من كلها كانت حاملة جين AmpC، وهي البلورات ذات خصائص أربعية أمنية من عائلة AmpC. تم بعد ذلك خضع لمضادات أربعة جينات من عائلة AmpC، وكانت نسبة لها blaFOX 8(44.4%) و blaCIT 7(38.9%) و blaDHA 3(17.9%) و blaEBC 9(50%) على التوالي. و المضادات الأخرى هو أ                       

152
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/mm³ 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 ≥10⁵ 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 an sterile 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°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 by AmpC 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 37°C. A positive 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.

**Multiplex PCR Mixture**

All blaAmpC 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 primers 10 pmole/μl (consist of forward 1.5 μl and reverse 1.5 μl for each primer) , DNA template 5μl 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

<table>
<thead>
<tr>
<th>Multiplex gene</th>
<th>Initial denaturation</th>
<th>Cycling condition</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature (°C)/Time</td>
<td>denaturation</td>
<td>annealing</td>
</tr>
<tr>
<td>blaFOX</td>
<td>94/3min</td>
<td>94/30 sec</td>
<td>64/30 sec</td>
</tr>
<tr>
<td>blaCIT</td>
<td>95/2min</td>
<td>95/30sec</td>
<td>61/30sec</td>
</tr>
<tr>
<td>blaEBC</td>
<td>95/2min</td>
<td>95/30 sec</td>
<td>62.6/30sec</td>
</tr>
<tr>
<td>blaDHA</td>
<td>95/2min</td>
<td>95/30 sec</td>
<td>61/30 sec</td>
</tr>
</tbody>
</table>

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 to April 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 polymorphonuclear pus cells/high power field). A total of 95 (73.08%) females and 35 (26.9%) males had positive urine culture with significant bacteriuria.

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

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

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 bacteriuria 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.
In the present study, an attempt was made to evaluate the frequency of \(\beta\)-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 \(\beta\)-lactam antibiotics were selected because they are the most commonly used antibiotics in the therapy of bacterial infections, compared to other \(\beta\)-lactam antibiotics. A part from their therapeutic usage, these antibiotics can provide a comprehensive primary screening of \(\beta\)-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).

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

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>No. of isolates</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>(E.) (coli)</td>
<td>46(76.7%)</td>
<td>14 (23.3%)</td>
</tr>
<tr>
<td>other Gram(-) and(+) isolates</td>
<td>54 (77.1%)</td>
<td>16 (22.9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100(76.9%)</td>
<td>30(23.1%)</td>
</tr>
</tbody>
</table>
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 3-3 and figure 3-4).
Figure (3-3): Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted DNA of blaAmpC positive E. coli isolates and amplified with blaCIT and blaFOX 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 blaCIT (462 bp) genes and Lanes (EC1, 4, 6, 11,13, 14, and 15) showed positive results with blaFOX (190 bp) genes.

Figure (3-4): Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted DNA of blaAmpC positive E. coli isolates and amplified with blaDHA and blaEBC 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 blaDHA (405 bp) genes and Lanes (EC1, 3, 4, 7, 8, 9, 10, 13, and 14) showed positive results with blaEBC (302 bp) genes.
The results in this study showed 8 (44.4%) had a bands compatible with blaFOX 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). whereas the CIT family (CMY-7, LAT14 and BIL-1) was presented in (38.9%) of blaAmpC 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 microorganism 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 blaDHA 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 blaEBC type gene. In comparison with other studies, reported that EBC enzyme was the most common gene (2157, 36.3%) among plasmid mediated β-lactamases producing enteric bacteria (32).

References:


