Microbiological and molecular study for detection of Uropathogenic 
*Escherichia coli* isolated from cases of urinary tract infection in Baghdad

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Abstract

Urinary tract infections are a major public health problem worldwideconstituting an estimate of 150 million cases per year. Uropathogenic E. coli isolates are the most common cause of community-acquired UTIs and responsible for the large portion of nosocomial UTIs. In this study, a total of 102 clinical samples were collected from a patient suffering from UTIs. UPEC has isolated on MacConkey and EMB agar, then further identified by a microscopic examination and API 20E. An antibiotic sensitivity test was done for 50 UPEC isolates that reveals a high rate of resistance to Aztreonam, Trimethoprim, Ciprofloxacin, and Tetracycline at ratio (76%, 74%, 66%, and 62%) respectively. PCR was used for detecting virulence Pap gene and more antibiotic resistance genes by using specific primers. From a total of 50 isolates, 46 isolates (92%) gave positive results for Pap gene and the amplified products was with a molecular size 328 bp while tet A gene was detected only in 31 isolates from a total of 50 isolates at ratio (77.4%) with a molecular size of 577bp, also only 37 (70.2%) of trimethoprim resistance isolates gave the amplified products of dfrA1 gene with a molecular size of 367 bp after electrophoreses on 2% agarose gel.

Keywords: UPEC, antibiotic sensitivity test, PCR

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Introduction

Urinary tract infection is considered as the most frequent human bacterial infection all over the world (1). The most common type of UTI is acute cystitis that is often referred to as a bladder infection or lower UTIs, also infection of the upper urinary tract or kidney known as pyelonephritis (2). Studies show that about 50% of the women and 12% of men get UTIs during their life (3). UTI is predominantly caused by the Colonization of the Gram-negative bacterial species such as *Escherichia coli*, *Klebsiella*, *Proteus* and *Pseudomonas* (4).

Uropathogenic *Escherichia coli* is one of the most prevalent organisms that infect the urinary tract and possess multiple virulence factors such as fimbriae, toxins, and siderophores which facilitate bacterial growth and persistence within the urinary tract (5). Expressions of adhesive factors like P pili allow UPEC to bind and invade to the host cells and tissues within the urinary tract. P fimbriae consist of heteropolymeric fibers composed of four protein subunits together with some accessory proteins; one major subunit, Pap A, and three minor subunits, Pap E, Pap F and Pap G (6). Pap A gene forms the bulk of protein structure and it’s essential for fimbriae formation. The minor subunits are minutely localized at the tip of the fimbrial structure with a more elaborate Pap C that found on the outer membrane, forming an assembly platform for fimbrial growth (7). These fimbriae components are arranged on a multicistronic gene cluster called pap that encoded by the pap gene operon (8). The ultimate UTI infection may be seen to comprise three distinct stages (1) bacterial attachment and colonization. (2). Local invasion. (3) disseminated systemic disease (9).

The management of UTIs has become increasingly challenging as a result of the emerging resistance of UPEC to most first-line antimicrobial agents that increased considerably due to mutation and horizontal gene transfer by plasmids or transposon (10). The high antimicrobial resistance of UPEC significantly reduces the therapeutic options and increases the treatment costs and mortality rates (11).
Material and methods

Samples collection
A total of urine samples (102) were collected from patients suffering from urinary tract infection from (3) hospitals in Baghdad, AL-Imameinkadhimein medical city, Al-Yarmouk teaching and Al-Kadhimiya pediatric hospitals during the period from 1/10/2016 to 1/1/2017. The samples were directly streaked on MacConkey agar, incubated at 37°C for 24 hrs, and then selected isolated colonies were subcultured on EMB agar and were further identified by gram stain and API 20E system.

Antibiotic susceptibility test
50 isolates were selected to be tested by the antibiotic sensitivity test that was performed using the Kirby-Bauer method according to CLSI, (2016) guidelines. Uropathogenic E.coli isolates were cultured in (5) ml of nutrient broth to reach turbidity equivalent to (0.5) Mcfarland tube containing approximately (2 X 108) CFU /ml then incubated at 37°C for 24hrs. After incubation, a sterile cotton swab was dipped in a nutrient broth, then streaked on Muller-Hinton agar medium with rotating the plate in any direction, then with a sterile forceps antibiotic disk was pressed gently on the agar surface, not more than (5) discs were put in each plate. Within (15) min of applying discs, the inverted plate was incubated at 37°C for 24 hrs. After the incubation period, the diameter of the inhibition zone was measured and compared to the standard value of each drug according to CLSI, (2016) published data.

Genetic study

Extraction of genomic DNA
Genomic DNA was extracted according to the Promega kit protocol and as follows:
3 ml of LB broth was inoculated with 2-4 colonies of E.coli from MacConkey agar then incubated at 37°C for 24hrs with shaking. 1ml of an overnight bacterial culture was put in an Eppendorf tube and centrifuged at 13000-16000 rpm for 2 minutes to pellet the bacterial cells, the supernatant was removed. 100 μl of freshly prepared lysozyme solution was added to the pellet, the supernatant was removed. Pellet was washed with D.W and centrifuged again. 600 μl of nucleic lysis solution was added, mix gently by inverting, incubated for 5 min at 80°C, and then cooled to room temperature. 3 μl of RNase Solution was added and mixed well, incubated at 37°C for 30 minutes, then cooled to room temperature. 200 μl of Protein Precipitation Solution were added to the DNA for the residual proteins, mixed by vortex and incubated on ice for 5 minutes, then Centrifuged at 13,000–16,000 rpm for 3 minutes. The supernatant was transferred to a clean Eppendorf tube contains 600 μl of room temperature isopropanol, mixed by inversion, centrifuged and decant the supernatant.600 μl of 70% ethanol at room temperature were added to the pellet and mixed, Centrifuged for 2min. Then aspirated the ethanol and air-drying the pellet for 10–15 minutes. Rehydrated the DNA pellet in 100 μl of Rehydration Solution for 1 hour at 65°C.

Estimation of DNA quantity
A nanodrop spectrophotometer was used to estimate the concentration and purity of the extracted DNA samples according to the following procedure:
Concentration (μg/ml) = (A260 reading–A320 reading) x dilution factor x50μg/ml

PCR Principle
To carry out a PCR experiment, four fundamental components are required (Brown, 2002). These components include:
DNA template containing the region of the DNA fragment to be amplified.DNA polymerase was used to synthesize a DNA copy of the region to be amplified. A pair is of oligonucleotide primers complementary to the DNA regions 5’ and 3’ ends of the DNA region that is to be amplified. A supply of deoxynucleotide triphosphates (dNTPs) from which the DNA polymerase builds the new DNA. PCR amplification of DNA involves three stages with alternating temperatures. In the first stage, at 94°C, DNA is completely denatured to single-stranded DNA. The second stage, at 40-70ºC, involves annealing of the target DNA by primers complementary to the 3’ ends of opposite strands of the DNA. In the third stage, at 72°C, DNA polymerase extends each primer, duplicating the target sequence. These three stages are repeated 30-40 times yielding new duplicates every cycle, resulting in many copies of the wanted DNA fragment, which are the PCR products. In this study Uropathogenic E. coli isolates were screened for the presence of pap virulence gene by amplification used a specific primer as in Table 1. As well as bacterial isolates which give resistant results for tetracycline and trimethoprim in antibiotic sensitivity test were further subject to molecular detection of tetA and dfr1A genes by PCR using specific primers as in Table 2.
Table 1: Primers of *pap* virulence gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Size of product (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pap</em></td>
<td>F: GACGGCTGTACTGCAAGGTGTCG&lt;br&gt;R: ATATCCCTTTCTGCAGGGATGCAATA</td>
<td>328</td>
<td>(Rahdar et al., 2014)</td>
</tr>
</tbody>
</table>

Table 2: Primers for antimicrobial *tetA* & *dfr1A* resistance genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Size of product (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>TetA</em></td>
<td>F: GGTTCACCTGAAAGCAGTCA&lt;br&gt;R: CTGTCCGACAAAGTTGCATGA</td>
<td>577</td>
<td>(Randall et al., 2004)</td>
</tr>
<tr>
<td><em>dfr1A</em></td>
<td>F: GGAGTGCCAAAAAGTGAAACGC&lt;br&gt;R: GAGGCGAAGTCTTGGGTAAGAAC</td>
<td>367</td>
<td>(Momtaz et al., 2012)</td>
</tr>
</tbody>
</table>

Optimal PCR cycling program condition

Table 3: PCR amplification condition for *pap* virulence genes

<table>
<thead>
<tr>
<th>Step</th>
<th>No. of cycle</th>
<th>Temperature – time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>1 cycle</td>
<td>94°C for 5 minute</td>
</tr>
<tr>
<td>Denaturation</td>
<td>35 cycle</td>
<td>94°C for 30 second</td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
<td>62°C for 30 second</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72°C for 30 second</td>
</tr>
<tr>
<td>Final Extension</td>
<td>1 cycle</td>
<td>72°C for 10 min.</td>
</tr>
</tbody>
</table>

Table 4: PCR amplification condition for *tetA* & *dfr1A* resistance genes

<table>
<thead>
<tr>
<th>Step</th>
<th>No. of cycle</th>
<th>Temperature – time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>1 cycle</td>
<td>94°C for 5 minute</td>
</tr>
<tr>
<td>Denaturation</td>
<td>35 cycle</td>
<td>94°C for 1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
<td>61°C for 1 min</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72°C for 1 min</td>
</tr>
<tr>
<td>Final Extension</td>
<td>1 cycle</td>
<td>72°C for 10 min.</td>
</tr>
</tbody>
</table>

Results and discussion

Bacterial isolates were identified by a microscopic examination and cultural characteristics on two selective media MacConkey and EMB agar. It gives dark pink colonies on McConkey due to lactose fermentation and green metallic sheen on EMB agar. Then UPEC isolates were identified by API 20 E system. They gave negative results in the Arginine Dihydrolase test (ADH), Citrate Utilization test (CIT), H2S production test, urease production test (URE), Tryptophan Deaminase test (TDA), Voges-Proskauer test (VP) and Gelatin Liquefaction tests (GEL). Isolates gave positive results in Indole Production test (IND), beta-lactamase test (ONPG), Lysine Decarboxylase test (LDC), Ornithine Decarboxylase test (ODC), glucose, mannitol, sorbitol, rhamnose, melibiose fermentation tests but gave negative results in sucrose, inositol, amylase fermentation tests.

Results revealed that only 76 (74.5%) isolates gave typical morphological characteristics and biochemical tests related to Escherichia coli. A study in Baghdad included the isolation a total of 143 urine samples from UTIs patients in the Diabetes Center of AL-Yarmouk Hospital revealed that *E. coli* was the most common cause of UTI in diabetic patients (28.6%) followed by *Klebsiella spp.* (20%) and *Proteus mirabilis* (18.5%).
Staph aureus (16.7%) (12). 507 urine samples were collected from both genders. The most common isolated pathogen was UPEC (82.6%), 64.5% of the isolates were from females while 35.5% of them were from males (13). In a study reported, 90 UPEC strains were isolated from patients with UTI for the period from 2008 to 2009. The ratio of male to female was 2:5 and the average age was 49 years (14).

**Antibiotic susceptibility test**

From a total number of bacterial isolates, 50 isolates were selected to be tested on the antibiotic sensitivity test. Uropathogenic isolates were found to exhibit different obvious levels of resistance and susceptibility patterns for these antibiotics. The diameter of the zone of inhibition for these isolates compared to the CLSI was illustrated as shown in Table (5).

Results of the antibiotic susceptibility test revealed that Imipenem was found to be one of the most effective antibiotics against UPEC that 100% of the tested isolates were sensitive to it. This result closely agrees with results recorded by (15) who found that only 2% of E.coli exhibited resistance to Imipenem that acts by the inhibition of peptidoglycan synthesis in bacterial cell walls through the inhibition of essential enzymes (transpeptidases) involved in the terminal stages of peptidoglycan biosynthesis. A study carried out in the Teaching Laboratories center in Baghdad revealed that (96%) of uropathogenic isolates were sensitive to Imipenem. Carbapenem was the most effective drug of choice against Gram-negative isolates (16).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>S</th>
<th>%</th>
<th>I</th>
<th>%</th>
<th>R</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>46</td>
<td>92</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>42</td>
<td>84</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>33</td>
<td>66</td>
<td>7</td>
<td>14</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>12</td>
<td>24</td>
<td>21</td>
<td>42</td>
<td>17</td>
<td>34</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>13</td>
<td>26</td>
<td>14</td>
<td>27</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>22</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>28</td>
<td>56</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>3</td>
<td>6</td>
<td>17</td>
<td>34</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>16</td>
<td>32</td>
<td>3</td>
<td>6</td>
<td>31</td>
<td>62</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>9</td>
<td>18</td>
<td>8</td>
<td>16</td>
<td>33</td>
<td>66</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>13</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>74</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>10</td>
<td>20</td>
<td>2</td>
<td>4</td>
<td>38</td>
<td>76</td>
</tr>
</tbody>
</table>

*S = Sensitive, I = Intermediate, R = Resistance.

Results of the antibiotic sensitivity test also revealed that 92% of isolates were sensitive to Nitrofurantoin and 84% of them were sensitive to Cefoxitin, also 66% of them were sensitive to Chloramphenicol. These results agreed with (17) who reported that 96% of UPEC isolates were sensitive to Nitrofurantoin. It showed a high percentage of sensitivity because it's reduced by bacterial flavoproteins to reactive intermediates compound and inactivate or alter bacterial ribosomal proteins and other macromolecules. So the vital biochemical processes such as protein synthesis, aerobic energy metabolism, DNA synthesis, RNA synthesis, and cell wall synthesis are inhibited. The broad-based nature of this antibiotic may explain the lack of acquired bacterial resistance to Nitrofurantoin.

UPEC isolates had a high rate of resistance to Aztreonam (76%), Trimethoprim (74%), Ciprofloxacin (66%) and Tetracycline (62%). They were a moderate rate of resistance to Streptomycin (60%), Gentamicin (56%), Ampicillin-sulbactam (46%) and Amoxicillin- clavulanic Acid (34%). UPEC isolates were found to give variable levels of resistance to ß-lactam antibiotics used in this study due to
bacterial secretion of β-lactamase enzyme encoded by their chromosome or plasmid and to prevent the inhibition of the bacterial cell wall synthesis. This result agreed with a study reported by (18) in Mexico who noted that 31.3% of UPEC strains isolated from UTIs were resistant to Amoxicillin-clavulanic acid, 75.9% of isolates were resistant to Ampicillin-sulbactam. The results also revealed that out of 50 tested uropathogenic. coli isolates, 37 isolates showed a high level of resistance (74%) to trimethoprim, and 31 isolates (62%) were developed resistance to tetracycline. This result agreed with (19) who noted that UPEC have a highly significant resistance to trimethoprim and with the results reported by (19) who revealed that (73.8%) of uropathogenic. coli isolates were resistant to tetracycline. Bacterial Cells have become resistant to tetracycline by enzymatic inactivation of tetracycline, efflux pump and ribosomal protection (20).

Genetic study

**pap** gene

PCR performed for 50 UPEC isolates in order to determine the presence of virulence *pap* gene encoded for pilin associated with pyelonephritis by using a specific primer, 46 isolates (92%) gave positive results and the amplified products was with a molecular size 328 bp that clearly appeared after electrophoresis on agarose gel as shown in Figure(1.1). P pilis of UPEC strains were encoded by the chromosomal *pap* gene cluster, present on the pathogenicity islands of UPEC strains. A study by (21) revealed that 85% of UPEC isolates were *pap* positive and *pap* gene was predominated in women suffering from UTIs of all ages. The percentage was ranged from 65% among patients from 18–30 years to 44% among patients from 51–70 years old.

![Figure (1.1): Gel electrophoresis for PCR product of *pap* gene. (Agarose 2%, 10min. at 100 voltage and then lowered to 70 volts, 60min.). Visualized under U.V light after staining with ethidium bromide. Line M: DNA marker (100-1000bp), Line 1-11: represented positive results of *E.coli* isolates that gave amplified product (328 bp), Line N: Negative control.](image)

Results from another study indicate that among UPEC isolated from patients with acute pyelonephritis and cystitis, who lack the underlying medical risk of infection, approximately 80% and 30% of them respectively possess P fimbriae (22). In another study in Mexico, clinically diagnosed women with UTIs were further screened to identify the presence of virulence genes, fimH was detected in (86.1%) of UPEC isolates and papC was in (62%) of them. The high incidence of the papC gene indicated that UPEC isolates have the ability to attach the kidneys cell and cause pyelonephritis (23).

Detection of antimicrobial resistance genes

**tetA** gene

PCR detection for tetA was performed under optimal conditions by using a specific primer. From a total of 50 extracted DNA samples, only 31 isolates that were previously exhibited resistance against tetracycline in antibiotic susceptibility test were used for screening the tetA gene by PCR. The results in Figure (1.2) showed that only (77.4%) of tetracycline resistance isolates gave amplified products of tetA that was present with a molecular size of 577bp after electrophoresis on 2% agarose gel.
**Figure (1.2):** Gel electrophoresis for PCR product of tetA gene. (Agarose 2%, 10min. at 100 voltage and then lowered to 70 volts, 60min.). Visualized under U.V light after staining with ethidiumbromide. LineM: DNA marker (100-1000 bp), Line 1-11: represented positive results of *E.coli* isolates that gave amplified product (577bp), Line N: Negative control.

Tetracycline resistance in bacteria is mediated by three strategies including limiting the access of tetracycline to the ribosomes, altering the ribosome to prevent effective binding of tetracycline, and producing tetracycline-inactivating enzymes. Limiting the access of tetracycline into the ribosomes may lead to the reduction in the intracellular concentrations of tetracycline through pumping the antibiotic out of the cell at a rate equal or greater than its uptake (24). Tet pumps are divided into six groups based on the amino acid sequences. The tetA gene is located on conjugative plasmids of different incompatibility groups. The resistance gene product is a cytoplasmic-membrane protein that is an energy-dependent tetracycline transporter (25).

One possible explanation for the presence of tet genes at different percentages among UPEC isolates is that the number of these isolates has a mosaic gene encoding an efflux pump and ribosomal protection protein which has recently been described in a number of human samples. A recent analysis of the (Tn1721) transposon has revealed a regulatory gene tetR encoding for a 26 kDa repressor protein and a structural gene tetA encoding a membrane protein 34 kDa that is responsible for resistance in UPEC (26). In Iran, a study reported by (27) who collected out of 210 urine samples from patients with UTIs, 150 isolates of UPEC were identified and all these isolates were tested for their sensitivity against tetracycline by disc diffusion method then PCR was used for the detection of the presence of tetracycline genes. The results demonstrated that (86.27%) of the isolates carried the tetA gene. Also, another study involved testing 123 isolates of Escherichia coli isolated from UTIs patients by disk diffusion method indicated that the UPEC isolates had a maximum resistance to tetracycline antibiotic (73.98%), then these isolates were subjected to PCR by using a specific primer. The distributions of tetA resistance genes were detected at a ratio of 43.80% (28). UPEC isolates recovered from clinical samples at three hospitals in Nigeria between June 2009 and May 2010 were investigated. The isolates were subjected to standard procedures of antibiotic susceptibility and polymerase chain reaction, the tetA gene was detected in (44%) of UPEC isolates (29).

**dfrA1 gene**

PCR technique was used to amplify a targeted dihydrofolatereductase gene which is responsible for trimethoprim resistance in UPEC isolates by using specific primer, from a total of 50 extracted DNA samples of UPEC, only 37 isolates that were previously exhibited resistance in antibiotic susceptibility test were used for screening *dfrA1* gene by PCR. The results in Figure (1.3) showed that only (70.2%) of trimethoprim resistance isolates gave the amplified products of *dfrA1* that was present with a molecular size of 367 bp after electrophoreses on 2% agarose gel.
Figure (1.3): Gel electrophoresis for PCR product of \textit{dfrA1} gene. (Agarose 2\%, 10min. at 100 voltage and then lowered to 70 volts, 60min.). Visualized under U.V light after staining with ethidiumbromide. Line M: DNA marker (100-1000 bp), Line 1-11: represented positive results of \textit{E.coli} isolates that gave amplified product (367bp), Line N: Negative control.

Trimethoprim affects the bacterial folic acid synthesis by inhibition of dihydrofolate reductase (DHFR), which catalyzes the reduction in dihydrofolate. Bacteria may become resistant to TMP by several mechanisms, including the development of permeability barriers, efflux pumps, and the existence of naturally insensitive target DHFR enzymes, also mutational and regulation changes in the target enzymes and the acquirement of drug-resistant target enzymes \cite{30}. Trimethoprim resistance in clinically gram-negative bacterial isolates is usually acquired by horizontally transferable of resistance genes (for genes) coding for alternative resistant dihydrofolate reductases. Such genes can be found as a gene cassettes carried by integrons that forming parts of transposons, which mediate to the widespread dissemination of trimethoprim resistance. The most common resistance mechanism is the acquirement of TM-insensitive DHFR genes, more than 30 different TM resistance \textit{dfr} genes are known and divided into two major types, pdf, and dry. Integron class 1 and class 2 have been shown to harbor \textit{dfr} gene cassettes (\textit{dfrA5}, \textit{dfrA7}, \textit{dfrA12}, \textit{dfrA17}, \textit{dfrA22}, and \textit{dfrA27}) that have been found within variable regions of class 1 integrons. \textit{dfrA1} and more recently \textit{dfrA14} have been shown to be associated with class 2 integrons \cite{31}. In Algeria, a study published by \cite{32} for the investigation of antibiotic resistance genes revealed that from a total of 150 community-acquired uropathogenic \textit{Escherichia coli} that were collected, only (65\%) of UPEC isolates were carried \textit{dfrA1} gene.

In conclusion, UPEC was predominant bacteria isolated from cases of urinary tract infections. The bacteria revealed high resistance to antibiotics. PCR reveals the presence of virulence pap gene in high percentage also tet A & \textit{dfrA1} resistance gene found in almost isolates.

References


