Detection of $tetM$, $armA$, $blaPER-1$ and $blaIMP$ genes in $E. coli$ isolates among the gram negative bacteria that cause urinary tract infections

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Abstract

The purpose of this study was to define the prevalence of gram negative bacteria that cause urinary tract infections (UTIs) and to look for the genes $tetM$, $blaIMP$, and NDM-1 in uropathogenic Escherichia coli isolates. This study was conducted on 205 urine samples collected from outpatients having a urinary tract infection for Baquba teaching hospital. The VITEK 2 compact device, which is fully automated, was used to identify the causative microorganisms and also used in antimicrobials susceptibility test. Combined-disk test was used for phenotypic determination of beta lactamases producing isolates and after DNA extraction, PCR was used to determine the genotypic status of the $16S rRNA$, $tetM$, $blaIMP$, and $NDM-1$ genes. Out of 205 urine samples, only 72(35.12%) were found to have gram negative bacteria, isolated microorganisms were $Escherichia coli$ 44 (35.7%) , $K. Pneumonia$ 18(14.6%) , $P. aeruginosa$ 8(6.5 %) were 2 (1.6 %) diagnosed as $Burkholderia cepacia$. Overall highest susceptibility was observed for imipenem, meropenem, amikacin, momocycline, colistin and to tazobactam and 32 isolates from $E. coli$ were confirmed to be ESBL producers. $16S rRNA$ gene sequencing attributed the isolates to $Escherichia coli$. Among the 10 $E. coli$ isolates recovered from the UTI patients, isolates which positive for $tetM$ (90%) and which negative for $armA$, $blaPER-1$ and $blaIMP$. The most common gram negative bacteria found to cause UTIs were $E. coli$ and $K. pneumoniae$. This study found that a few pathogens of $E. coli$ are resistant to IPM and MEM. The frequency of $tetM$ genes that are responsible for this resistance among pathogenic $E. coli$ isolates in diyala, was high.

Keywords: Uropathogens, Drug resistance, VITEK system, $Escherichia coli$, PCR.
Introduction

Urinary tract infection refers to any infection of the urinary tract, which includes the urethra, bladder, ureter, and kidneys, caused by pathogenic microorganisms(1). Urinary tract infection is one of the most common forms of bacterial infection, as studies have shown that infection affects 150 million people annually worldwide, and that 47% of people have infection at least once during their lifetime(2). Bacteria are the main cause of Urinary tract infection among other microorganisms such as viruses, fungi, and parasites (3). Gram-negative bacteria are the main cause of infection, at a rate of 75%, and gram positive bacteria also cause infection, but at a rate of less than 25%(4,5).

Resistance to at least three antibiotics from different groups, such as aminoglycosides, chloramphenicol, and tetracyclines, is known as MDR of bacteria (6,7 ). Antibiotic resistance among uropathogens is becoming a growing concern. Depending on the degree of resistance, the site of infection, and the availability of safe, nontoxic therapeutic alternatives, drug resistance may have a major clinical impact (8).

Bacteria can resist antibiotics through a variety of mechanisms, including degradation or modification of the antibiotic; alteration of the antibiotic's bacterial target; target protection; and reduction of the antibiotic's intracellular concentration, either by decreased cell wall permeability or through efflux of the antibiotic from the cell. In comparison to the other known pathways, efflux-mediated resistance has been overlooked. Many bacterial efflux pumps will expel several, unrelated groups of antimicrobial compounds from the cell, fostering multidrug resistance phenotypes (9,10).The current study aims to detect gram negative bacteria that cases urinary tract infection with multiple resistance to antibiotics by bacteriological and biochemical methods and to determine the ESBL and by phenotype and genotype methods

Materials and Methods

This cross study was conducted in Baquba teaching hospital between from August in 2020 to February in 2021. The study included all urine specimens which were collected from both sexes. The sample of clean catch mid-stream urine sample was collected in "a sterile universal container and processed as per standard microbiological techniques"(11). All urine specimens was examined by microscope and only specimens have infection cultured on blood agar and MacConkey agar plate. Primary identification of bacteria isolates were based on microscopical, cultural, morphological diagnosis and biochemical tests, while the confirmatory identification test was based on VITEK® 2 Compact device and antibiotic susceptibility testing was performed also by VITEK® 2 Compact device. The apparatus contains special data which convert result of bacterial metabolism to bionumber, the calculation to the system compare this number with blank and give a fast result during 8hrs.

Phenotypic Detection of E.coli ESBLs production

The capacity of bacteria to generate ESBLs was determined using the combined disc process. The bacterial suspension was prepared and separated on Petri dishes containing Muller Hinton agar and left for 10 minutes to be dried. A disc containing combination of Amoxicillin / Clavulanic acid (30 mg / Disc) was affixed in the center of the inoculated plate. Then the antibiotic discs of Tazobactam , cefuroxime and cefoxitin were arranged on distance of 3 cm of the disc in the center and after incubated at 37C for 24 hours the observation of incorporation of inhibition zone between the disc in the center and one or more of antibiotic discs around referred to a positive result.
efflux pumps activity

The EtBr-agar cartwheel (EtBrCW) method is a useful tool for detecting increased efflux activity in large groups of clinical isolates from various bacterial species. This method helps to compare various isolates based on their ability to extrude EtBr. Bacterial isolates were cultured overnight in a shaker with NB before being modified to 0.5 of a McFarland standard(12) the next day. The NA plates were divided into a cartwheel pattern by radial lines. The bacteria were then swabbed (using a sterilized cotton swab) onto NA plates containing increased concentration of EtBr and incubated for 16 hours at 37°C. A UV transilluminator was used to study the culture on the NA plates. Isolates that emit fluorescence at higher concentration of Ethidium Bromide were considered to have more active efflux system than isolates that emit fluorescence at low concentration of Ethidium Bromide.

Genotypic detection of E.coli isolates

DNA Extraction, In this study DNA extraction kit method was successful and efficient for extraction, all extracted DNA was intact and pure. The extracted DNA was detected by electrophoresis on Agarose gel 1%, stained with ethidium bromide, electrophoresed in 70 volt for 1hr and photographed under ultraviolet (UV) transilluminator

PCR reaction, Specific primers were used to amplify the sequences of the 16S rRNA, tetM, blaIMP, armA and blaPER-1 genes(Table 1). The PCR assay was carried out in a total volume of 50 µl of mixture, which included 25 µl master mix, 4 µl DNA sample, 4 µl forward primer, 4 µl reverse primer, and 13 µl ddH2O, as shown in Table 2. Table 3 shows the PCR timetable program for the 16S rRNA, tetM, blaIMP, and NDM-1 genes. Once analyzed by 2% agarose gel electrophoresis, the PCR products were stained with ethidium bromide and photographed.

Table 1 : Primers used in the current study.

| No. | Gene     | Sequence of forward and reverse Primer(5’ - 3’) | Product bp | Origin |
|-----|----------|-----------------------------------------------|------------|--------|
| 1   | 16S RNA  | F ATGACGTCAGTCATCAGTGAGG <br> R AGCGAGGTATCCAGCCGCA | 353 (13)   |        |
| 2   | tetM     | F GTGGACAAAGGTACAACGAG <br> R CGTAAAAGTTCGTACACAC | 406        | This study |
| 3   | blaIMP   | F GAAAGGCGTTTATGTTGTTAC <br> R GTAAGTTTCAAGAGTGATGC | 587        | This study |
| 4   | armA     | F CAAATGGGATAAGATGATGTT <br> R CCATCCTCTCTCTCTTTCCA | 646 (14)   |        |
| 5   | blaPER-1 | F CCGCGGATCTGGAACCTTT <br> R TGTCCTCTTGTTGTTTTC <br> R TGACACTTTATCAGCAACC | 513        | This study |

Table 2 : Components used in the current study

| Components        | Concentration | Volume (50 µl) |
|-------------------|---------------|----------------|
| 2X PCR Taq Master Mix | 1X            | 25 µl          |
| Forward primer    | 10 µM/µl      | 4 µl           |
| Reverse primer    | 10 µM/µl      | 4 µl           |
| ddH2O             | -             | 13 µl          |
Table 3: Programs of PCR thermo cycling conditions used in the current study.

| Phase             | Tm (°C) | Time       | Cycles |
|-------------------|---------|------------|--------|
| "Initial denaturation" | 94°C    | 5 min      | 1X     |
| "Denaturation"    | 94°C    | 30 sec.    | 35X    |
| "Annealing"       | 56°C    | 30 sec.    |        |
| "Extension"       | 72°C    | 1 min      |        |
| "Final extension" | 72°C    | 5 min      | 1X     |

Results

After a general urine examination done for urine sample, it was found that 205 patients had acute urinary tract infection: 123 (60 %) with bacterial positive culture, 82 (40 %) with bacterial negative culture and biochemical test among all isolates show that gram negative bacteria was the most prevalent bacteria 72 isolates (58.5%) while the gram positive bacteria 51 isolates (41.4%) (table 4).

Table (4) : the number of urinary tract infection, showing the sample type and their percentages

| Sample type | Total |
|-------------|-------|
|             | No.   | %    |
| Positive culture |       |      |
| G - ve       | 72 (58.5%) |
| G + ve       | 51 (41.4%) |
| Negative culture | 82     | 40.0 |

According to the results of the current study’s cultural, microscopically, and biochemical tests, 72 different gram-negative bacterial isolates from 205 urine samples of patients with urinary tract infection were found that constituting from 44 isolates (35.7%) Escherichia coli, 18 isolates (14.6%) were K. Pneumonia, 8 isolates (6.5%) diagnosed as P. aeruginosa, 2 isolates (1.6 %) diagnosed as Burkholderia cepacia table (5).

Table (5) : Distribution outpatients infected with urinary tract infection according to type of isolate.

| Type            | Bacterial Isolates | Total |
|-----------------|--------------------|-------|
|                 | No.    | %    |
| G – ve bacteria | E. coli  | 44    | 35.7 |
|                 | Klebsiella pneumoniae | 18    | 14.6 |
|                 | Pseudomonas aeruginosa | 8     | 6.5  |
|                 | Burkholderia cepacia | 2     | 1.6  |

Most bacterial isolates were highly resistant to most antibiotics, particularly piperacillin and Ticarcillin, according to the results of antibiotic susceptibility tests. Meropenem and Imipenem provided the best antibacterial effect against most isolates. The best antimicrobials for Gram-
negative organisms \((n = 72)\) were imipenem\((18.0\%)\) , meropenem \((16.6\%)\) , amikacin \((22.2\%)\) , momocycline \((20.8\%)\) , colistin\((22.2\%)\) and to tazobactam \((27.7\%)\) and moderate resistance rate were ciprofloxacin \((33.3\%)\) , ceftazidime \((40.2\%)\) , cefepime \((31.9\%)\) , aztreonam \((47.2\%)\) , gentamicin \((33.3\%)\) , tobramycin \((40.2\%)\) , Pefloxacin\((47.2\%)\) and trimethoprim \((38.8\%)\) ; however, the high resistance rate was found to be against ticarcillin \((79.1\%)\) and piperacillin \((83.3\%)\) as in table \((6)\).

Table \((6)\) : Antimicrobial susceptibility testing of gram negative bacteria isolated from the urine of outpatients with a urinary tract infection

|          | Gram negative bacteria 72 (100) |
|----------|---------------------------------|
|          | S(100%) | I(100%) | R(100%) |
| TIC      | 15(20.8) | 0(0)    | 57(79.1) |
| PIP      | 11(15.2) | 1(1.3)  | 60(83.3) |
| TZP      | 46(63.8) | 6(8.3)  | 20(27.7) |
| CAZ      | 35(48.6) | 8(11.1) | 29(40.2) |
| FED      | 41(56.9) | 8(11.1) | 23(31.9) |
| ATM      | 34(47.2) | 4(5.5)  | 34(47.2) |
| IPM      | 59(81.9) | 0(0)    | 13(18.0) |
| MEM      | 60(83.3) | 0(0)    | 12(16.6) |
| AN       | 51(70.8) | 5(6.9)  | 16(22.2) |
| GM       | 41(56.9) | 7(9.7)  | 24(33.3) |
| TM       | 43(59.7) | 0(0)    | 29(40.2) |
| CIP      | 48(66.6) | 0(0)    | 24(33.3) |
| PEF      | 32(44.4) | 6(8.3)  | 34(47.2) |
| MNO      | 54(75)   | 3(4.1)  | 15(20.8) |
| CS       | 54(75)   | 2(2.7)  | 16(22.2) |
| SXT      | 44(61.1) | 0(0)    | 28(38.8) |

An isolate was resistant to at least three of the antimicrobial agents tested, it was classified as multidrug resistant (MDR) \((16)\). The multidrug resistance phenotype of Gram negative bacteria was shown \(55.5\%\) as show in figure \((1)\).

Figure 1 : MDR, XDR, and PDR Gram-Negative bacteria were isolated from outpatients with a urinary tract infection. MDR: Multidrug resistance; XDR: Extensive drug resistance; PDR: Pandrug resistance. The multidrug resistance phenotype of Gram negative bacteria was shown by the *Escherichia coli* \(63.6\%\), *Klebsiella* species \(38.8\%\), *Pseudomonas aeruginosa* \(57.1\%\), *Burkholderia Cepacia* \(50\%)\) as show in table \((7)\).
Table (7): Numbers and percentages of MDR, XDR and PDR of gram negative bacteria isolated from urine of outpatients infect with urinary tract infection.

| Resistance       | MDR  | XDR  | PDR  |
|------------------|------|------|------|
| E. coli          | 28(63.6%) | 1(2.2%) | 0(0.0%) |
| K. pneumonia     | 7 (38.8%) | 0(0.0%) | 0(0.0%) |
| p.aeruginos      | 45(57.1%) | 0(0.0%) | 0(0.0%) |
| Burkhoderia Cepacia | 1(50%) | 0(0.0%) | 0(0.0%) |
| Total            | 40 (55.5%) | 1(1.3%) | 0(0.0%) |

**Phenotypic detection of E.coli isolates**

Extended spectrum β-lactamase enzyme production it was used to detect the isolates ability to produce ESBLs enzyme. The results showed that 32 (72.7 %) isolates ESBLs enzyme producer, as show in table(8).

Efflux pump activity, the activity of efflux system in the isolates were determined by Ethidium Bromide agar CartWheel method (EtBrCW). The results showed that 37(84.1%) isolates have the efflux pump, as show in table (8). The efflux system might be largely responsible for intrinsic resistance of some E. coli isolates to antibiotics(17).

Table (8): Numbers and percentages of phenotypic results Gram-Negative bacteria (E.coli) isolated from outpatients infect with urinary tract infection

| phenotypic results (E.coli) | Total 44 (100%) | P value |
|-----------------------------|-----------------|---------|
| ESBL                        | 32 (72.7%)      | 0.003** |
| efflux pump activity        | 37 (84.1%)      | 0.0001**|

**Genotypic detection of E.coli isolates**

DNA Extraction, In this study DNA extraction kit method was successful and efficient for extraction, all extracted DNA was intact and pure. The extracted DNA was detected by electrophoresis on Agarose gel 1%, stained with ethidium bromide, electrophoresed in 70 volt for 1hr and photographed under ultraviolet (UV) transilluminator.

Molecular diagnosis of E. coli Isolates

The results of all 10 samples were identified as Escherichia coli as shown in table (9) and Figure(2) depend on 16S rRNA gene. In this study the detection of 16SrRNA gene was 100%, showed that 16SrRNA gene is used for accurate diagnosis because it is one of the genes to other species because if a steady sequence of each bacterial species has important role in molecular identification and Classification (18).

Table (9): Number and percentage of positive isolates for presence of screened 16S rRNA, tetM, armA, blaPER-1 and blaIMP genes of ten E.coli isolates

| Strain       | 16S rRNA | tetM | armA | blaPER-1 | blaIMP |
|--------------|----------|------|------|----------|--------|

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Figure (2): Gel electrophoresis detect 16S rRNA gene (353 bp) using DNA Ladder (1k) promega.

**Virulence factor gene profiles of E. coli isolates**

In current study, when the virulence gene profiles are evaluated and 3 types of virulence profiles are identified. In total, 60% of the strains carry at least one virulence gene. The study demonstrated that E. coli isolates which positive for each of tetM (90%) and which negative for each armA, blaPER-1 and blaIMP, as show in table (9) and figures (3).

![Table](null)

| SN01 | SN02 | SN03 | SN04 | SN05 | SN06 | SN07 | SN08 | SN09 | SN10 | Total |
|------|------|------|------|------|------|------|------|------|------|-------|
| +    | +    | -    | -    | -    | +    | -    | +    | -    | +    | 10 (100%) |
| +    | +    | -    | -    | -    | +    | -    | +    | -    | +    | 9 (90%)   |
| +    | +    | -    | -    | -    | +    | -    | +    | -    | +    | 0 (0%)    |
| +    | +    | -    | -    | -    | +    | -    | +    | -    | +    | 0 (0%)    |
| +    | +    | -    | -    | -    | +    | -    | +    | -    | +    | 0 (0%)    |

Figure (3): Gel electrophoresis detect tetM gene (406 bp) using DNA Ladder (1k) promega.

**Discussion**

The study found that the number of isolates that gave a positive result for bacterial culture was 123 isolates (60.0%). While the number of isolates that gave a negative result was 82 (40.0%) as shown in Table (4), this result in disagreement to results of (19) as the percentage was (32.6%). The most common pathogenic bacteria responsible for UTIs were discovered to be E. coli and K. pneumonia with a percentage of isolation reached 35.7% and 14.6% respectively as illustrated in table(5).
*E. coli* was the most prevalence bacteria (44 isolates 35.7%) (table 5), previous studies (20) in Erbil governorate show that *E. coli* reported the most prevalence bacteria 41 isolates (58.5 %). this result are in agreement with previous study ( 21). A variety of virulence factors specific for colonization and invasion of the urinary epithelium, such as P-fimbria and S-fimbria adhesions, are a significant contributing factor for *E. coli* isolating at a higher rate(22).This result are disagreement with previous study (23) reported that *Staphylococcus aureus* was the most prevalence bacteria (40.4 %) followed by *E. coli* (31.8%).This study revealed that *Klebsiella pneumoniae* was the second most common bacterial ( 18 isolates , 14.6 %) isolated from urine samples from patients follows *E. coli* (5) , this result in agreement with previous studies ( 24,25).

*Klebsiella pneumoniae* is considered one of the most enteric bacteria constitutes a serious problem in urinary tract infection, that similar to (26) and (21) reported that from a total of specimens were collected from patients with UTI *Pseudomonas aeruginosa* represents ( 8 isolates , 6.4 % ) from the total isolates, Earlier studies (25,27,28).reported different rates of *Pseudomonas aeruginosa* (2.8 % ) , (23.4%), and (5.55%), respectively. *Burkholderia cepacia* represents ( 2 isolates , 1.6%) from the total isolates. Li et al. previously described a case of Burkholderia urinary tract infection in renal transplant recipients who needed graft nephrectomy (29). Gram negative isolates had a higher prevalence rate of resistance to widely prescribed antibiotics, according to the current study. The etiology of bacteria that cause UTIs, as well as their resistance to antimicrobials, has changed over time and differs between countries (30,31). Extended spectrum fourth generation antibiotics (e.g. cefepime) have a higher resistance to enzymatic degradation by -lactamases (especially AmpC -lactamases) and a better ability to penetrate Gram negative bacteria's outer membrane porins (32).

Repeated use of antibiotics antibiotics may harm peri-urethral flora, enabling uropathogens to colonize and invade the urinary tract, leaving clinicians with little options of drugs for UTI care. Furthermore, this condition allows bacteria to exchange genetic material through horizontal gene transfer, resulting in resistant genes that confer antibiotic resistance (33,34). MDR can be caused by inadequate and inaccurate antimicrobial agent administration as empirical therapy, as well as a lack of effective infection control methods, resulting in an increase in the prevalence of resistant organisms in the population (35,36).

Since the 1980s, beta-lactam antibiotics (penicillins, cephalosporins, carbapenems, and monobactams) have been commonly used to treat serious infections caused by Gram-negative bacteria, leading to the rapid spread of bacteria resistant to these antibiotics around the world (37,38).

Beta-lactamases hydrolyze beta lactam antibiotics, which is the most common mechanism of resistance for this class of antibiotics in Gram-negative bacteria (39).

The tetM gene was determined in 90% of the *E. coli* isolates included in this study. The results of the current study showed that *E. coli* isolates negative for the armA gene. This study is an approximation of a previous study( 40) showed that 5.4% of isolates have armA gene. previous study showed that armA seems to have high prevalence is Enterobacteriaceae producing NDM (New Delhi Metallo-β-lactamase)-type carbapenemase (41).

The study demonstrated that *E. coli* isolates which negative for blaIMP gene. This result disagreed with the study of (42) showed that 14.58 % of isolates have NDM-1 gene. The rapid spread of Carbapenem-resistant *Enterobacteriaceae* is due to the clonal and plasmid-mediated dissemination of clinical carbapenem-resistant strains (43).

Extended spectrum -lactamase (ESBL) producing and carbapenem resistant E. coli has increased globally over the last decade (44). A varying number of different mechanisms are thought to be involved in the resistance to carbapenems. Primarily, the process includes the
production of carbapenemases like class A KPC, class B metallo-β-lactamases (IMP, VIM and NDM) as well as class D OXA-type enzymes (OXA-48-like) (45).

Carbapenem resistance may also be caused by AmpC type enzymes or ESBLs, as well as membrane impermeability (46). Modifications or absence of OmpC and/or OmpF porin channels, as well as the presence of drug efflux pumps, can cause membrane impermeability (47).

**Conclusion**

The results of the current study showed that the prevalence of *E. coli* and *K. pneumonia* were to be the greatest common pathogenic bacteria responsible for UTI. The results suggest that the prevalence of MDR for gram negative bacteria is distressingly high. Antibacterial resistance patterns have to be updated periodically to confirm proper empiric treatment of UTI. Our results showed that the frequency of antibiotic resistance genes of tetM and NDM-1 is very high in *E. coli* strains isolated from patients in Baquba teaching hospital.

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