Multiplex PCR amplification for the detection of Biofilm and extended-spectrum Beta-Lactamase resistance genes and antibiotic resistance patterns in uropathogenic *E. coli*

Abstract:

The issue of urinary tract infections, particularly those stemming from Escherichia coli belonging to the Enterobacteriaceae family, has received considerable critical attention and is evaluated as the second most common infection in humans. Uropathogenic *Escherichia coli* (UPEC), which is virulent, produces extended spectrum beta-lactamase (ESBL), as well as being multidrug-resistant (MDR), is considered to be a common growing public health issue worldwide. This could be a contributing factor for UTIs to become far more severe, first-line antibiotics to become less effective, and thus cause increase in morbidity and mortality to rates. In this experiment, 73 *Escherichia coli* strains have been isolated from urine specimens. The antibiotic susceptibility of the isolates was evaluated through the disc agar diffusion method. Taken together, the resistance pattern is the underlying basis of MDR. Three significant biofilm genes and antimicrobial resistance mechanisms were evaluated in these isolates with ten typical antibiotic discs, considering that the data was processed using SPSS statistical software, version 25. During the course of this investigation, 73 isolates of *E. coli* were examined, in which *pap* gene is present in 89% of isolates, *fim*H gene is present in 86.3% of isolates, and *sfa* gene is present in 69.9% of isolates. Furthermore, the beta-lactamase gene *bla*SHV, *bla*TEM, and *bla*CTX-M, gene frequency were 50.7%, 90.4%, and 79.5 respectively. The results regarding antibiotic resistance pattern elucidated that a significant number of the isolates were resistant to Imipenem, Amoxicillin, and Ampicillin respectively. This study suggests that the formidable rate of virulent ESBL-producer *E. coli* strains used in such experiment compels the performance of an antibiotic stewardship program, regional surveillance regarding extended-spectrum beta-lactamase (ESBL)-producing organisms and their associated virulence determinants for the purpose of logical antibiotic selection, or developing novel UTI treatment strategies by inactivating the essential virulence factors relating to UPECs.

Keywords: UPEC, *E. coli*, ESBL, UTIs, Biofilm

1. Introduction:

Uropathogenic *E. coli* (UPEC) is routinely associated with urinary tract infections (UTIs), particularly among female cases. It is probable therefore that numerous virulence factors (VFs) found in UPEC isolates have an impact on the frequency as well as severity of UTIs (1,2). A number of these VFs contain genes that encode adhesins, including temperature-sensitive hemagglutinin (*tsh*), *papA*, curli fimbriae (*csgA*), papC-associated pili, a fimbrial adhesin (*afa*), and the type-1 fimbriae (fimH) play a substantial role in the adherence of bacteria to the urinary tract, facilitating the initial stages of urinary tract infections (UTIs). Additionally, genes encoding

toxins such as α-hemolysin (hlyD) exhibit detrimental impacts on the host and significantly accelerates the pathogenicity of such bacterial strains (3,4). Genes which encode invasins such as *ibeA* take part in the invasion of microvascular endothelial cells in the brain, while genes that encode protective factors including increased serum survival (*iss*) help protect *E. coli* from the bactericidal enterprise of serum. Moreover, genes that encode siderophores like the *sitA* iron transporter facilitate bacterial growth, and the pathogenicity island marker *malX* is involved in the glucose and maltose transportation in terms of treating urinary tract infections. The escalating prevalence of antibiotic resistance sets a significant health risk (5,6).

Bacterial communities known as biofilms adhere to surfaces and are enclasped by an extracellular polymeric substance (EPS) matrix. Such biofilms form in response to environmental signals, allowing a population of bacteria to grow and survive in the urinary tract. In controlled laboratory settings, a specific strain of *E. coli* has been shown to produce biofilms with varying characteristics based on factors such as nutrient composition, temperature, and flow dynamics (7). Additionally, autotransporter proteins that facilitate bacterial aggregation and adhesion, as well as the synthesis of exopolysaccharides (e.g. cellulose) that form the biofilm matrix encompassing the bacteria, play a role in this process (8). The process of adherence between bacteria and host cells is facilitated by specific bacterial adhesins such as P-fimbriae, which are expressed by the pyelonephritis associated pili gene or the *pap* gene. P-fimbriae has an indispensable function in the colonization of the upper urinary tract by bacteria, their affixation to the renal vascular endothelium, and the subsequent development of pyelonephritis (9). Another significant adhesion factor to consider is Sfimbriae, as it is controlled by either the S-fimbrial adhesion gene or the *sfa* gene. These genes fall under the category of mannoseresistant adenosine and are situated within a region of the chromosome known as Pathogenicity Islands (10). The *sfa* and *pap* genes are frequently observed in *E. coli* strains isolated from urinary tract infections (UTIs) and are responsible for encoding pili that aid in the adherence of bacteria to the host tissues, resulting in the formation of antibiotic-resistant biofilms. It is crucial to identify UPEC strains capable of producing biofilms in order to gain a deeper understanding of the pathogenicity and antibiotic resistance mechanisms exhibited by this bacterium in UTIs (11).

The study findings indicate that beta-lactam antibiotics are commonly prescribed for treating urinary tract infections in clinical settings (12). Certain bacterial strains, notably those of Escherichia coli, are capable of producing extended-spectrum beta-lactamases (ESBLs), enzymes that can degrade penicillins, first to third-generation cephalosporins, and monobactams (aztreonam). Clavulanic acid, tazobactam, or sulbactam have been demonstrated to effectively inhibit the three primary ESBL enzyme groups - TEM, CTX-M, and SHV (13). Notably, managing infections resulting from ESBL-producing *E. coli* can prove to be increasingly difficult and restricted in cases where resistance to additional antibiotic categories, including aminoglycosides, tetracyclines, chloramphenicol, trimethoprim-sulfamethoxazole, and fluoroquinolones, is present concurrently, often found within the same plasmids containing the ESBL genes. This particular occurrence has the potential to significantly impact the expenses associated with morbidity and the rate of mortality among individuals with UTIs (14). Hence, the study is focused on examining the prevalence of biofilm genes (*pap*, *fimH*, and *sfa*) alongside extended-spectrum Beta-Lactamase

resistance genes (*bla*TEM, *bla*CTX-M, and *bla*SHV), as well as investigating the antimicrobial resistance profiles of UPEC in Karaj, Iran.

2. Materials and Methods:

2.1. Sample collection and Isolation of *E. coli*

This investigation was meticulously planned with a focus on 78 *E. coli* isolates found in the urine samples of outpatients suspected of UTIs, spanning a period of 9 months from October 2022 to June 2023. Yielded 73 E. coli isolates from which 59 of the 73 samples were drawn from female and 14 from male subjects. These samples were transported in less than two hours to the lab after being placed in the TSB transport medium. Moreover, these samples were cultured on Blood agar (Ibresco, Iran), MacConkey agar (Ibresco, Iran), and EMB agar (Ibresco, Iran) plates incubated for a duration of 24 hours at 37° C. Subsequently, strains of *E. coli* were obtained by standard microbiological methods.

2.2. Antimicrobial Susceptibility of *E.coli* isolates

Samples had been chosen in order to examine the susceptibility patterns of the 10 antimicrobial agents belonging to various classes. Tetracycline (30µg), Imipenem (10µg), Ampicillin (10µg), Co-trimoxazole (25µg), Amikacin (30µg), Cefixime (5 µg), Cefalexin (30µg), Amoxicillin (25µg), Ciprofloxacin (5µg), and Gentamicin (10µg) discs (Padtan Teb, Iran). The susceptibility of *S. aureus* isolates to antibiotics was investigated using the Kirby-Bauer method, with analysis based on the guidelines outlined by CLSI.

2.3. DNA extraction

DNA was extracted from samples using the boiling method. A loopful of bacterial colonies were suspended in 300 µl of sterile distilled water and subsequently subjected to heating for a duration of 20 minutes. The liquid part was used as a DNA sample in the PCR mixture after spinning it in a machine for 15 minutes at a fast speed of 13,000 rpm (15).

2.4. Detection of genes

PCR and electrophoresis techniques were used to determine the *pap*, *fimH*, *sfa*, *blaTEM*, *blaCTX-M*, and *blaSHV* genes. The *pap*, *fim*H, and *sfa* genes were subjected to the following amplification conditions: denaturation for five minutes at 95°C, 30 cycles of 95°C for 1 minute, 56°C for 40 seconds, 72°C for 45 seconds, and the last extension for 5 minutes at 72°C (15–17). The *bla*TEM, *bla*SHV, and *bla*CTX-M genes were amplified given the following circumstances: A preliminary denaturation at 95°C for 15 minutes, followed by 30 amplification cycles. Each cycle included denaturation at 94°C for 30 seconds, annealing at 62°C for 90 seconds, and elongation at 72°C for 60 seconds. An ultimate elongation step at 72°C for 10 minutes was then carried out (18–20). The molecular approach was optimized by *E. coli* ATCC 25922 as the control positive strain. The primer sequences and PCR conditions required to detect genes are displayed in Table 1.

Table 1. Primers sequences as per standard reference

Gene	Primer Sequences (5′ to 3′)	Product Size (bp)	annealing	Reference
pap	F: GCAACAGCAACGCTGGTTGCATCAT R: AGAGAGAGCCACTCTTATACGGACA	336	56°C	15
*fim*H	F: GAGAAGAGGTTTGATTTAACTTATTG R: AGAGCCGCTGTAGAACTGAGG	559	56°C	16
sfa	F: CTCCGGAGAACTGGGTGCATCTTAC R: CGGAGGAGTAATTACAAACCTGGCA	410	56°C	17
blaCTX-M	F: ATGTGCAGYACCAGTAARGTKATGGC R: TGGGTRAARTARGTSACCAGAAYCAGCGG	593	62 °C	18
blaSHV	F: CTT TATCGGCCCTCACTCAA R: AGGTGCTCATCATGGGAAAG	237	62 °C	19
blaTEM	F: CGCCGCATACACTATTCTCAGAAT GA R: ACGCTCACCGGCTCCAGATTTAT	445	62 °C	20

2.5. Statistical analysis

The correlation between sociodemographic factors and the frequency of *E. coli* isolation was ascertained using model selection log-linear analysis on categorical data. Additionally, the statistical analyses were all conducted by means of the SPSS 25.0 software for Windows. The threshold for statistical significance was determined to be $P < 0.05$.

3. Results:

3.1. Antibiotic susceptibility

The cumulative resistance of *E. coli* isolates to antimicrobial agents was 95.9 % for Ampicillin; 84.9% for Imipenem, 57.9% for Amoxicillin and 32.9% for Cefalexin. Specifically, the findings suggests that the highest sensitivity was related to Amikacin (86.3%), Gentamicin (78.1%), and Co-trimoxazole (75.3%), respectively. The overall antibiotic susceptibility pattern of strains to antimicrobial agents is shown in Table2. Figure 1 presents the percentage of antibiotic resistance by gender.

Table 2. Antimicrobial susceptibility pattern of *E. coli* in total isolates.

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)
Tetracycline	41 (56.2)	11 (15.1)	21 (28.8)
Amikacin	63 (86.3)	2 (2.7)	8 (11)
Ampicillin	1 (1.4)	2 (2.7)	70 (95.9)
Amoxicillin	37 (36.8)	9 (5.3)	27 (57.9)
Cefalexin	39 (53.4)	10 (13.7)	24 (32.9)
Co-trimoxazole	55 (75.3)	6 (8.2)	12 (16.4)
Cefixime	41 (56.2)	14 (19.2)	18 (24.7)
Imipenem	8 (11)	3 (8.2)	62 (84.9)

Ciprofloxacin	43 (58.9)	9 (12.3)	21 (28.8)
Gentamicin	57 (78.1)	1 (1.4)	15 (20.5)

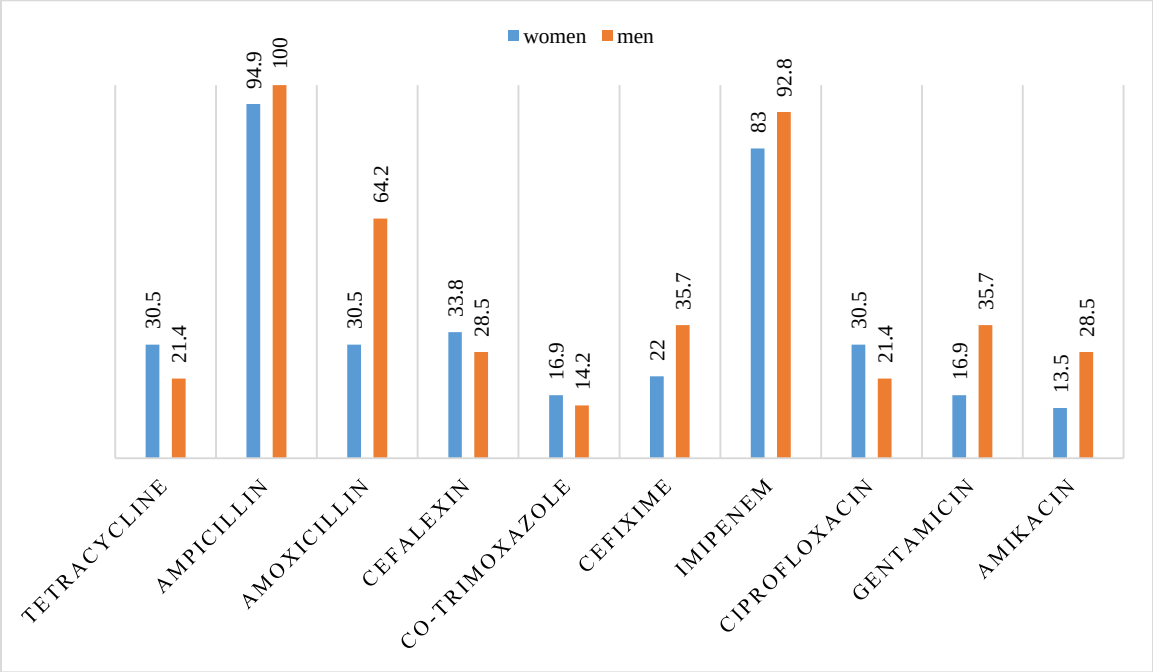

Figure1. The percentage of antibiotic resistance by gender.

3.2. Gene pattern characterization

The findings obtained from this investigation demonstrate that the occurrence of biofilm-producing genes in urine samples was as follows: the *sfa*, *fim*H, and *pap* genes was 69.9%, 86.3%, and 89% respectively. Also, the frequency of β-lactamase genes in clinical samples was as follows: *bla*SHV, *bla*TEM, and *bla*CTX-M genes was 50.7%, 90.4%, and 79.5% respectively. PCR product of genes from *E. coli* isolates is show in Figure 2 and 3.

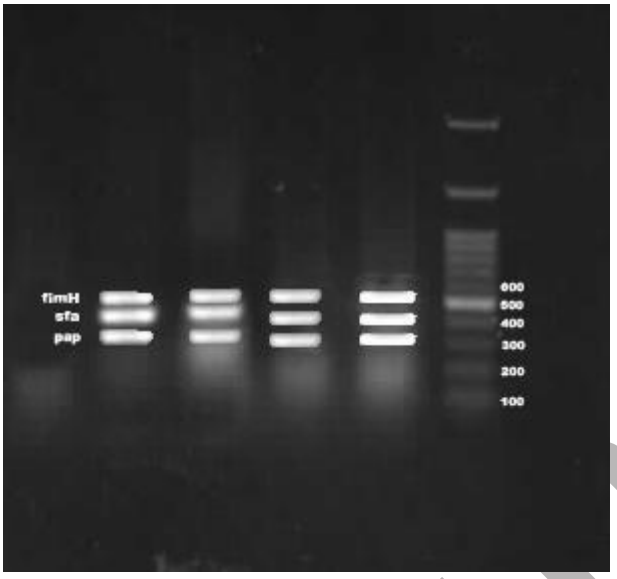

Figure2. Multiplex PCR Amplification of *pap*, *fimH*, *sfa* from *E.coli* isolates. 100bp DNA ladder.

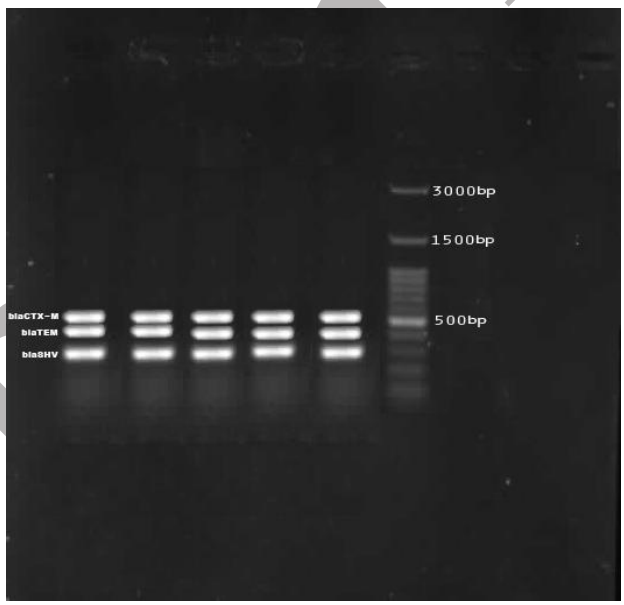

Figure3. Multiplex PCR Amplification of *bla*SHV, *bla*TEM, and *bla*CTX-M genes from *E.coli* isolates. 100bp DNA ladder.

A notable discrepancy was noted in the occurrence regarding biofilm genes (*pap* and *fim*H) in relation to gender ($P<0.05$). The statistical analysis disclosed noteworthy associations between the resistantly against Amoxicillin ($P=0.04$) and tetracycline ($P=0.05$) of the blaSHV gene in the UPEC isolates. Furthermore, it indicated significant connections between the resistantly against Amoxicillin ($P=0.02$) and Gentamicin ($P=0.04$) of the *bla*CTX-M gene, along with critical associations between Ampicillin ($P=0.001$) and *bla*TEM in the UPEC isolates.

4. Discussion:

Studies confirm that *E. coli* is the most prevalent factor in UTI occurrence among both ambulatory individuals and those hospitalized. Such disparity may be attributed to differences in anatomical structure between the male and female genital tracts, particularly because of the fact that urethra in females is shorter, allowing easier access for pathogenic species such as *E. coli* to reach the bladder. In terms of microbial expansion, the position of the female urethral orifice, which is close to the vaginal as well as anal area, contributes to the less demanding action by the microbes reaching the urinary tract area (21).

In the current investigation, a complete set of 73 isolates belonging to the E. coli species were examined, in which *pap* gene is present in 89% of isolates, *fim*H gene is present in 86.3% of isolates, and *sfa* gene is present in 69.9% of isolates. Also, the beta-lactamase gene *bla*SHV, *bla*TEM, and *bla*CTX-M gene frequency were 50.7%, 90.4%, and 79.5 respectively. The results belonging to the antibiotic resistance pattern showed that most of the isolates were resistant to Ampicillin, Imipenem, and Amoxicillin respectively.

In a report by Yazdi et al. (22), The frequency of the *fim*, *pap*, and *sfa* genes was 100%, 79%, and 69% respectively, which was close to the results of the current investigation. In another major study, Saki et al. (23), stated the prevalence of biofilm genes *pap*, *fim*H, and *sfa* as 96.6%, 93.3%, and 4.6%, respectively, among which the prevalence of *fim*H and *pap* genes are close to this paper's results. In study of Naziri et al. (24), within the ESBL-producing strains, 100% were found to harbor *bla*CTX-M genes, 63% possessed *bla*SHV genes, and 11.1% carried *bla*TEM genes.

Additionally, Habeeb et al. (25) found that 42.5% of isolates contained the blaCTX-M gene and 48.1% contained the blaTEM gene. Tiba et al. (26) conducted a study on 162 isolates of uropathogenic Escherichia coli in Brazil, reporting frequencies of 27.8% for the pap gene and 6.2% for the sfa gene. In 2006, Arisoy et al. (27). Published a paper in which 161 isolates of uropathogenic *Escherichia coli* were examined and the frequency of *sfa* and *pap* genes were calculated as 6.2% and 28.9%, respectively. Cristea et al. (28) observed that 19.7% of *E. coli* isolates contained the gene *bla*SHV. Additionally, Sadeghi et al. (29) noted that 38.8% of isolates carried the gene *bla*CTX-M.

Similar studies that have been done, show that the prevalence of resistant *E. coli* is highly influenced by the geographical area and biological patterns which means that the prescription manner of common antibiotics for such bacterial infections varies from one certain area to another, which leads to diverse study outcomes around the world. Therefore, investigating the change of antibiotic resistance pattern in specific periods can be very efficient in the treatment of UTIs infections. To begin with organization of pathogenicity and urinary contamination, uropathogenic *Escherichia coli* strains must interface with the target location and fimbriae qualities play a critical role in this association. According to the show, it appears that out of the three examined qualities, the *fim*H and *pap* quality had the most noteworthy recurrence in this ponder. Since Urinary tract infection ranks highly as a matter of prevalence within the realm of healing center contaminations, it is essential to conduct thorough research on anti-microbial resistance due to the overuse of antimicrobials in order to develop a vaccine against such fimbria.

Data availability

All findings derived or examined throughout the course of this investigation have been encompassed within the confines of this published article.

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None.

Authors' Contribution

literature review and research, writing-reviewing and editing, supervision, methodology, project administration, conceptualization, studies analysis, investigation: A.S.B., S.H.N., N.S., Writing original draft preparation, writing-reviewing and editing, and methodology: N.S., N.O., investigation. Validation and Reviewing: M.V., M.M.

Ethics

Not Applicable.

Conflict of Interest

The authors acclaim no conflict of interest.

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