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Research Article

Rapid Detection of *Escherichia coli* and *Klebsiella pneumoniae* in Urinary Tract Infections Using Multiplex PCR assay

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ABSTRACT

Escherichia coli and *Klebsiella pneumoniae* predominantly cause urinary tract infections (UTIs). Rapid and accurate identification of these pathogens is crucial for effective treatment. This study aimed to evaluate multiplex PCR for the simultaneous detection of *E. coli* and *K. pneumoniae* in urinary samples. Conducted in 2023 at Ghaem Hospital, Mashhad, Iran, 250 urine samples from suspected UTI patients were collected. DNA was extracted using a commercial kit and amplified using specific primers (*EC-16S* for *E. coli* and *KP/khe* for *K. pneumoniae*). Results were compared with culture methods. Among the 250 samples, 110 were positive: 61 *E. coli*, *49 K. pneumoniae*, and 12 co-infections. Multiplex PCR showed high sensitivity and specificity with faster turnaround than culture. It is a reliable and rapid diagnostic tool for UTIs. Multiplex PCR offers a rapid, sensitive, and specific alternative to culture for UTI diagnosis, enabling timely treatment decisions.

1. Introduction

Urinary tract infections (UTIs) are among the most prevalent bacterial infections worldwide, affecting individuals across all age groups and genders. These infections represent a significant public health concern due to their high prevalence, frequent recurrence, and potential for serious complications if not treated promptly (Jhaveri *et al*., 2024). It is estimated that more than 30% of the global population is affected by UTIs at some point in their lifetime (Amedina & Castillo, 2019). Urinary tract infections (UTIs) are among the most common bacterial infections in Iran, with a reported overall prevalence of approximately 13.3% based on national meta-analyses (Ghafari *et al*., 2016). In a local study conducted at Imam Reza Hospital in Mashhad, more than 60% of urine samples from hospitalized patients were culture-positive, indicating a significant burden of UTI in this region (Moradi *et al*., 2021). Therefore, the implementation of efficient and rapid methods for the interpretation of urine samples by laboratory personnel is essential for achieving standardized and reliable diagnostic outcomes (Zimoń *et al*., 2024). Among the wide spectrum of urinary pathogens, *Escherichia coli* and *Klebsiella pneumoniae* are the most commonly isolated gram-negative bacteria responsible for both community-acquired and nosocomial UTIs (Hatrongjit *et al*., 2022). Due to the clinical

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relevance of these pathogens, it is crucial to adopt diagnostic strategies that enable their prompt identification. Conventional diagnostic techniques, including urine culture and biochemical assays, are widely accepted as the gold standard; however, they are inherently time-consuming and labor-intensive, typically requiring 24 to 48 hours to deliver definitive results (Foxman, 2014). This delay may postpone the initiation of appropriate antimicrobial therapy, potentially worsening patient outcomes. The increasing prevalence of multidrug-resistant (MDR) strains of *E. coli* and *K. pneumoniae* has further underscored the urgent need for rapid, accurate, and reliable diagnostic tools (Khameneh *et al*., 2019; Muthukrishnan *et al*., 2013). Molecular diagnostic methods, particularly those based on polymerase chain reaction (PCR), have revolutionized microbial detection by offering rapid, sensitive, and specific identification of pathogens (Kaye *et al*., 2024). In this context, multiplex PCR—capable of simultaneously amplifying multiple target genes in a single reaction—emerges as a powerful tool for the detection of multiple microorganisms with reduced consumption of samples and reagents (Gorgan & Shakeri, 2021). The present study aims to design and evaluate a multiplex PCR assay for the simultaneous detection of *E. coli* and *K. pneumoniae* directly from clinical urine isolates. This approach has the potential to enhance diagnostic efficiency and contribute to the timely and effective management of urinary tract infections, especially in the face of escalating antimicrobial resistance.

2. Materials and Methods

2.1. Sample collection

This cross-sectional study was conducted using urine samples collected from 250 patients (148 females and 102 males), aged between 7 and 70 years, who were suspected of having urinary tract infections. Inclusion criteria for patients suspected of having a urinary tract infection were symptoms such as burning sensation in urination, urgency in urination, or positive urine test findings such as leukocyte esterase/nitrite, and no antibiotic use. Samples were collected during the year 2023 from Ghaem Hospital in Mashhad, Iran. A 5 mL aliquot of each urine sample was transported under sterile conditions, maintained on ice packs, to the Microbiology Laboratory of Mashhad Islamic Azad University for further processing. The ethics code with the identification namber ID IR.IAU.MSHD. REC.1401.030 was obtained.

2.2. Sample processing method

A volume of 0.01 mL from each urine sample was inoculated onto eosin methylene blue (EMB) agar using a calibrated sterile loop. Plates were incubated under standard aerobic conditions (typically at 37°C for 24 hours). Following incubation, the plates were examined for colony morphology, growth characteristics, and the number of colonies. Colonies were further subjected to macroscopic and microscopic examination and differential biochemical tests for preliminary identification. The oxidase test, motility and indole test (SIM), trisaccharide iron agar (TSI), methyl red test, and Voges-Proskauer test (MRVP) were used to examine biochemical characteristics (CLSI) (Sharifian *et al*., 2023).

2.3. Microscopic analysis

The purpose of this method was to quantitatively assess the bacterial content of urine samples. In the hospital laboratory, 5 mL of urine was centrifuged at 4000 rpm for 5 minutes. A portion of the resulting sediment was transferred onto a sterile glass slide using a sampler and examined microscopically under 40x magnification. Normal urine was characterized by a low bacterial count, with 1–2 white blood cells (WBCs) and epithelial cells per field. An increased number of WBCs was indicative of a urinary tract infection.

2.4. DNA extraction

Genomic DNA was extracted from the urine samples using the Sinapure Gram-Negative Bacteria DNA Extraction Kit (SinaClon, Iran), following the manufacturer's protocol. Briefly, 5 mL of each urine sample was centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded, and the resulting pellet was used for DNA extraction. The quality and quantity of extracted DNA were assessed using agarose gel electrophoresis and a NanoDrop

spectrophotometer. DNA extraction was performed for all 250 urine samples.

2.5. Primer Design and Synthesis

Two primer sets were used in this study. The first targeted the *16S rRNA* gene (*EC-16S*) of *Escherichia coli* (GenBank Accession No. NR_024570.1) and was designed using Primer3 software (version 0.4.0). The second set targeted the *KP/khe* gene specific to *Klebsiella pneumoniae*, and its sequences were obtained from a published article. All primers were synthesized by SinaClon (Iran). Primer specificity was confirmed using BLASTn analysis against the NCBI nucleotide database. Analytical sensitivity was evaluated through ten-fold serial dilutions of genomic DNA extracted from standard strains. The limit of detection (LOD) was estimated to be approximately 100 bacterial cells per reaction. Positive controls consisted of genomic DNA from confirmed *E. coli* (ATCC 1330) and *K. pneumoniae* (ATCC 10031) strains. PCR-grade water was used as the negative control. The details of the primers used in this study are presented in Table 1.

Table 1. Characteristics of species-specific primers in the multiplex PCR reaction

Target Gene	Primers Sequences 5′–3′	Amplicon Size (bp)	Reference
EC-16S-F EC-16S-R	AACCTGGGAACTGCATGCATCTGA CAGCACCTGTCTCACGGTTC	433	(In this study)
KP/khe-F KP/khe-R	GATGAAACGACCTGATTGCATTC CCGGGCTGTCGGGATAAG	77	(Hartman *et al*., 2009)

2.6. PCR (Polymerase chain reaction)

Initial optimization of PCR conditions was performed using monoplex PCR assays to detect *Escherichia coli* and *Klebsiella pneumoniae* separately. The *KP/khe* and *EC-16S* primers were used to amplify target genes from standard strains. Each reaction was performed in a final volume of 25 µL using a commercial color master mix (SinaClon, Iran). The monoplex PCR was repeated under various reagent concentrations and thermal cycling parameters to optimize amplification conditions. A temperature gradient ranging from 51°C to 58°C was used to determine the optimal annealing temperature. The final optimized PCR conditions were as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds, and extension at 72°C for 45 seconds; followed by a final extension at 72°C for 4 minutes. The amplified PCR products were subjected to agarose gel 1% electrophoresis to confirm successful gene amplification (Hartman *et al*., 2009).

2.7. Multiplex PCR Assay

To simultaneously detect the presence of *K. pneumoniae* and *E. coli* in urine samples, a multiplex PCR assay was developed using the previously optimized conditions. An annealing temperature of 53°C was selected based on the monoplex optimization results. The multiplex PCR assay was performed in a total reaction volume of 25 µL, containing 12.5 µL of 2× PCR master mix (including Taq polymerase, dNTPs, $MgCl_2$, and buffer), 2 µL of template DNA, 0.75 µL of each forward and reverse primer (for *Escherichia coli and Klebsiella pneumoniae*), and 7.5 µL of nuclease-free distilled water. PCR products were electrophoresed on a 1% agarose gel stained with a suitable DNA dye, and bands were visualized under UV illumination. A nuclease-free distilled water was included as the negative control. Standard reference strains, *E. coli ATCC 1330* and *K. pneumoniae ATCC 10031*, were used as positive controls. A molecular weight marker (DNA ladder) was included in each run to estimate product sizes. Gel documentation was performed using a Gel Doc imaging system (Festa *et al*., 2023).

2.8. Statistical analysis

Statistical analysis was performed using SPSS software (version 26). Descriptive statistics were used to summarize the data. A

one-way analysis of variance (ANOVA) was employed to evaluate the agreement between multiplex PCR and conventional urine culture results. Statistical significance was defined as $p \leq 0.05$.

3. Results

Out of the 250 urine samples analyzed using multiplex PCR, *Escherichia coli* was detected in 61 samples (24.4%), *Klebsiella pneumoniae* in 49 samples (19.6%), and both bacteria were simultaneously detected in 12 samples (4.8%) (Table 2). Among the PCR-positive cases, 60% were female and 40% were male, with an age range between 23 and 60 years.

Of the 128 samples tested negative for *E. coli* and *K. pneumoniae* by multiplex PCR, 78 were also negative by conventional culture, corroborating the absence of bacterial infection in these specimens. The remaining 50 culture-positive samples harbored other uropathogens not included in the scope of the multiplex PCR assay.

All PCR products yielded clear, distinct bands corresponding to the expected sizes when visualized on 1% agarose gels. No nonspecific amplification or primer-dimer formations were observed, indicating high specificity of the assay. A representative gel electrophoresis image is shown in Figure 1.

Multiplex PCR detected a total of 122 positive cases (48.8%) for either or both pathogens, whereas the culture method identified 110 positive samples (44%). A discrepancy of 4.8% was observed between the two methods. Considering conventional culture as the gold standard, supplementary methods, including Nanodrop spectrophotometry (to assess DNA concentration) and urine microscopy, were employed to interpret questionable PCR results more accurately.

Nanodrop analysis generally confirmed sufficient DNA concentrations; however, in samples with high glucose or protein content, the results were less reliable. Urine microscopy was particularly useful in correlating cellular content with PCR band intensity and in resolving ambiguous findings. Positive samples consistently exhibited visible DNA bands with varying clarity, correlating with bacterial load and confirming results from culture-based methods.

Notably, bacterial counts below 10^5 CFU/mL in urine are generally considered within the range of normal flora. PCR results were negative for samples with bacterial counts of approximately 10^2 CFU/mL, while samples with 10^3–10^5 CFU/mL exhibited distinct bands, with band intensity increasing proportionally to the bacterial concentration.

Table 2. Distribution of detected bacterial species by multiplex PCR (n =250)

Detected Organism (s)	Number of samples	Percentage (%)
E.coli	61	24.4
K.pneumoniae	49	19.6
E.coli and K.pneumoniae	12	4.8
No detection by PCR	128	51.2

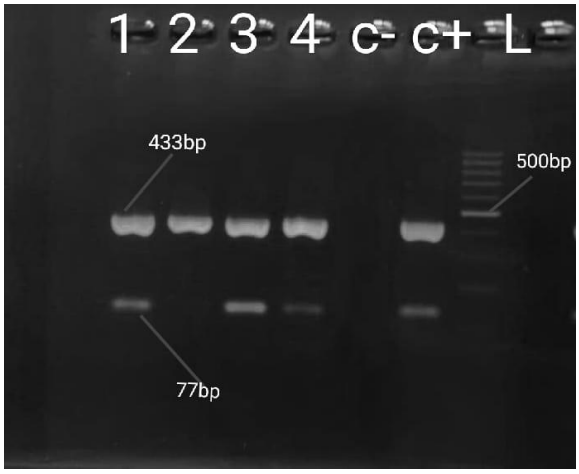

Figure 1. Electrophoresis of Multiplex PCR products of urine samples using two primers *EC-16S* and *KP/Khe*. Well 2: urine sample positive for *K. pneumoniae* and wells 1, 3, and 4: urine samples simultaneously positive for *E. coli* and *K. pneumoniae*, C+: positive control, C-: negative control, L: Ledder

4. Discussion

In recent years, PCR-based molecular diagnostics have gained prominence for their ability to rapidly and accurately detect infectious agents. Traditional culture-based methods, though considered the gold standard, require extended incubation times, typically 24 to 48 hours, and are prone to false positives or negatives due to the growth of environmental contaminants or fastidious organisms (Festa *et al*., 2023). Moreover, in culture media, the competitive overgrowth of non-target bacteria may mask the presence of uropathogens, thereby complicating diagnosis and delaying appropriate treatment.

In contrast, multiplex PCR allows for the simultaneous detection of multiple pathogens within a single reaction, enhancing efficiency, accuracy, and turnaround time. In the present study, two primer pairs (*KP/khe for K. pneumoniae* and *EC-16S for E. coli*) were employed for the identification of uropathogens directly from urine samples. Among 250 clinical samples collected from Ghaem Hospital, multiplex PCR identified *E. coli* in 24.4% of cases, *K. pneumoniae* in 19.6%, and co-infections in 4.8% of samples. These findings were consistent with the hospital laboratory's results and confirmed via electrophoresis.

Several recent studies have highlighted that PCR-based methods outperform conventional urine culture in detecting uropathogens. For instance, Wolfe et al. in a study published in MDPI on patients with complex UTIs, found that PCR identified 36.4% more organisms than culture, was 20 times more likely to detect polymicrobial infections, and 36 times more likely to identify fastidious organisms. Moreover, PCR detected 62.4% of organisms not found by culture, whereas culture detected only 9.4% of those identified by PCR (Wolfe *et al*., 2022).

A systematic review and meta-analysis conducted by Wang et al. demonstrated that molecular methods, including PCR and next-generation sequencing (NGS), exhibit significantly higher sensitivity and are capable of detecting a broader spectrum of uropathogens compared to conventional culture. While the analysis confirmed the superior diagnostic performance of these molecular approaches, it also emphasized that current evidence regarding their impact on clinical outcomes remains limited (Wang *et al*., 2022).

Consistent with these findings, the present study also showed that multiplex PCR was able to identify more uropathogenic organisms than conventional culture, highlighting its potential as a rapid and sensitive diagnostic tool in clinical microbiology.

Importantly, a 2023 study compared multiplex PCR with both standard and expanded quantitative urine culture (EQUC): PCR detected 395 organisms, of which 89.1% were confirmed by EQUC, whereas standard culture found only 27.3%. This confirms that PCR

mainly detects viable pathogens (Festa *et al*., 2023).

Finally, in a 2024 randomized trial on complicated UTIs, PCR-guided management led to better clinical outcomes (88.1% vs. 78.1%) and significantly shorter turnaround time (~49.7 h vs. 104.4 h) compared to conventional culture-guided care (Lai *et al*., 2024).

Our multiplex PCR identified *E. coli* (24.4%), *K. pneumoniae* (19.6%), and co-infection in 4.8% of samples. These rates align with prior findings where molecular diagnostics uncovered polymicrobial infections and higher detection rates (Festa *et al*., 2023). Consistent with van der Zee et al reports, PCR assay delivers results in hours versus 24–48 h for culture, reinforcing its clinical utility (van der Zee *et al*., 2022).

The multiplex PCR approach employed in this study offers several key advantages over conventional diagnostic methods for urinary tract infections. Most notably, it significantly reduces turnaround time—delivering results within 4 to 6 hours—compared to the 24-48 hours required for standard urine culture, and is even faster than traditional singleplex PCR assays. This method also demonstrated high sensitivity and specificity, with a detection limit of approximately 10^3 CFU/mL. No nonspecific amplification or primer-dimer formation was observed during gel electrophoresis, underscoring the assay's analytical accuracy. Moreover, its streamlined workflow—allowing simultaneous detection of multiple pathogens in a single reaction—minimizes reagent use, reduces hands-on processing time, and ultimately lowers overall diagnostic costs, making it highly suitable for routine clinical implementation.

Nevertheless, this study has certain limitations. The assay was restricted to detecting two major uropathogens *E. coli* and *K. pneumonia* which, although highly prevalent, do not represent the full spectrum of possible causative agents. In addition, the relatively modest sample size may not fully reflect the microbial diversity of UTIs in the studied population. Finally, while the PCR method offers excellent diagnostic performance, it does not provide antibiotic susceptibility data, which remains essential for guiding appropriate therapy. Future research incorporating broader pathogen panels and resistance gene detection is warranted to expand the clinical utility of this approach.

Conclusion

This study evaluated the effectiveness of multiplex PCR in detecting *E. coli* and *K. pneumoniae* in urinary tract infection (UTI) samples, comparing the results with conventional culture methods. Multiplex PCR successfully identified *E. coli* in 24.4%, *K. pneumoniae* in 19.6%, and co-infections in 4.8% of the tested samples. The method demonstrated superior sensitivity and a significantly faster turnaround time compared to culture. These findings underscore the utility of multiplex PCR as a rapid and accurate diagnostic tool for UTI pathogens. Although multiplex PCR does not provide antimicrobial susceptibility information, it serves as a valuable complementary tool alongside culture, particularly in clinical scenarios where rapid diagnosis is critical. Incorporating multiplex PCR into diagnostic workflows could improve infection management, reduce diagnostic delays, and minimize inappropriate antibiotic use. Further research is needed to evaluate its cost-effectiveness and impact on clinical outcomes in routine practice.

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Conflict of interest

The authors declare no conflict of interest related to this study.

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