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ORIGINAL ARTICLE

Development and Evaluation of Recombinase-Aided Amplification Assay for Rapidly Detecting *Escherichia coli* in Urine

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SUMMARY

Background: ***Escherichia coli*** **is the main pathogen of urinary tract infection (UTI). Early and rapid detection for UTI pathogens is still limited. The aim of this study was to develop and evaluate a recombinase-aided amplification (RAA) assay for rapidly detecting *Escherichia coli* in urine.**

Methods: **RAA specific primers and probes were designed based on the conserved region of *E. coli chuA* gene. The optimal primers and probe combinations were screened and identified using a real-time fluorescent RAA assay. The specificity was assessed by detecting *E. coli* and other none-*E. coli* uropathogens. The limit of detection (LOD) was determined through serial dilution. One hundred thirty-five midstream urine samples were used to evaluate the sensitivity, specificity, and accuracy. RAA and urine culture were performed in parallel. Urine culture is still the gold standard.**

Results: **The optimal combination of 2 primers with 1 probe was determined based on the real-time fluorescence RAA curves. The LOD of this method is 10^3 cfu/mL. No cross-reactivity was observed with 12 other uropathogens that are commonly encountered in UTI. Among the 135 urine culture results, 50 of them were *E. coli.* 75 of them were non-*E. coli*. The remaining 10 were culture negative. They were detected by both RAA assay and urine culture in parallel. The sensitivity, specificity and accuracy of RAA assay were 94.00%, 91.76%, and 92.59%, respectively.**

Conclusions: **The real-time fluorescent RAA assay for urinary *E.coli* established in this study is a highly sensitive, specific and cost-effective method. It can be utilized for rapid detection of urinary *E.coli* within 30 minutes.**
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KEYWORDS

urinary tract infection, *Escherichia coli, chuA* gene, recombinase-aided amplification

INTRODUCTION

Urinary tract infection (UTI) is one of the most common infections worldwide, with high morbidity, mortality, and economic burdens [1,2]. Women are more vulnerable to UTIs, and over 60% of them will experience at least one UTI during their lifetime, with the recurrence rates of 20% - 30% [3]. The epidemiology and species distribution of uropathogens varied among different regions [4]. Previous studies showed that *Escherichia coli* was the main pathogen of UTI, accounting

for 70% - 80% of uropathogens [5,6]. Among those patients with *E. coli* bacteriuria, 15% of them had *E. coli* bacteremia [7]. Thus, early and rapid detection of urinary *E. coli* is of great importance for UTI initial and empirical therapy.

The diagnosis of UTI and subsequent antibiotic treatments rely heavily on urine culture, followed by bacterial identification and antimicrobial susceptibility testing. However, it is time consuming and usually takes 2 - 4 days to get the results, which may delay initial and empirical antimicrobial therapy. The molecular diagnostic methods based on nucleic acid amplification, such as PCR and fluorescence real-time PCR, offer more sensitive and accurate detection of these pathogens [8,9]. However, they require sophisticated operation, expensive machines and a specialized laboratory. It is better to do batch testing instead of detecting the samples on arrival.

Recombinase-aided amplification (RAA) by Qitian (Wuxi, China) is able to amplify and detect target DNA at constant temperatures of 37℃ - 40℃ within 30 minutes. It is a rapid, specific, and cost-effective method for pathogen identification [10]. The amplification products could be detected either in real-time or at the end of amplification. Real-time detection does not require the lid to be opened during or after amplification, thus it could significantly reduce contamination, which is better than endpoint chromogenic stripe observation. Upon amplification, fluorophores and quenchers are released from probe, thus fluorescence signals could be detected [11]. RAA does not require PCR equipment, thus it could be performed in resource limited regions and in the area of point-of-care testing (POCT). The RAA method has been used to detect various pathogens, including bacteria, viruses, and parasites [12-14].

To the best of our knowledge, few studies have reported RAA assay used in detecting *E. coli* in urine. Based on the advantages of RAA techniques and the demand for rapid detection of urinary *E. coli*, we aim to establish a rapid RAA detection method to meet the need for early and rapid detection of UTI main pathogen *E. coli*.

MATERIALS AND METHODS

Bacterial strains and clinical samples

Bacterial strains used in this study were preserved in our laboratory. They included ATCC reference strains and clinical strains from urine samples as follows: *Escherichia coli* ATCC35218, *Enterococcus faecalis* ATC C29212, *Klebsiella pneumoniae* ATCC700603, *Enterococcus faecalis* clinical strain 1, *Klebsiella pneumoniae* clinical strain 1, *Acinetobacter baumannii* clinical strain 1, *Acinetobacter baumannii* clinical strain 2, *Pseudomonas aeruginosa* clinical strain 1, *Pseudomonas aeruginosa* clinical strain 2, *Enterococcus faecalis* clinical strain 1, *Enterococcus faecalis* clinical strain 2, *Enterobacter cloacae* clinical strain 1, *Enterobacter cloacae* clinical strain 2.

A total of 135 midstream urine samples, ordered for urine culture, were included at Peking University First Hospital from February 2024 to April 2024. Among them, 50 samples got positive urine culture results of *E. coli*, and 75 samples got positive urine culture results of non-*E.coli*. 10 samples were urine culture negative. This study was approved by the ethics committee of Peking University First Hospital, with the reference number No. 2023-293.

RAA primers and probes

Conserved sequences of *E. coli chuA* gene were screened based on database comparisons. Primers and probes were designed and analyzed for specificity based on the conserved sequence of *chuA* gene using NCBI primer-BLAST. Secondary structures and non-specific amplification of probes and primers were checked using Oligo7 software (http://www.oligo.net). Finally, 3 forward primers and 3 reverse primers were selected and screened. They were listed in Table 1.

The exact positions of probe and primers were labeled in the *E. coli chuA* gene (Figure 1). Forward primers were underlined in yellow, and reverse primers were underlined in purple. Probe was labeled in green. Two conserved thymine remnants within the probe were labeled with FAM-dT and BHQ1-dT with blue triangles. Primers and probe for HPLC-grade purification were synthesized at Tianyi Huiyuan Biotechnology (Beijing, China).

RAA assay

The urine sample of 10 mL was centrifuged at 3,000 g for 10 minutes. Supernatant was discarded and sediment transfered into a 1.5 mL Eppendorf tube. Then, 100 μL deionized water was added to sediment and vortexed to mix. The tube was transfered to a metal bath at 100℃ for 10 minutes, and then centrifuged at 12,000 g for 5 minutes. The supernatant is the extracted DNA solution. A commercial RAA kit (Qitian, Wuxi) was used. Briefly, 25 μL reaction buffer was added into 200 μL RAA reaction tube containing reaction powder, followed by 14 μL DNase-free water, 2 μL primer F (10 μM), 2 μL primer R (10 μM), 2 μL DNA template, and 1 μL probe (10 μM). This was then mixed and briefly centrifuged to collect the liquid at the bottom of the tube. Then, 4 μL magnesium acetate solution was added to the inside lid of the tube and centrifuge briefly. The total volume is 50 μL. Finally, a fluorescence detector was used to measure the fluorescence for 25 minutes at 39℃. Positive RAA results were judged based on whether there was significant elevation in the fluorescence amplification curve.

Determination of specificity

One strain of *E. coli* and 12 strains of other non-*E. coli* urinary pathogens described above (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterobacter cloacae*) were determined by RAA assay

and repeated in triplicate. DNA solutions of *E. coli* and other common uropathogens were repared as described above. The concentrations were adjusted to 10^9 CFU/mL.

Limit of detection (LOD)

E. coli ATCC35218 was cultured and its DNA was extracted. The DNA concentration was measured as 62.6 ng/μL using a Nanodrop instrument (ThermoFisher Scientific). The copy count was calculated to be 10^7 copies/μL according to the formula. The *chuA* gene is a single copy gene, 1 copy/μL = 1 CFU/μL. Thus, the concentration of DNA solution is 10^{10} CFU/mL, a 10-fold serial dilution of the DNA solution was made into different concentration gradients (10^9 - 10_3 CFU/mL). These serial solutions were used as templates in RAA to determine the LOD.

Diagnostic performance of RAA compared with urine culture

In order to evaluate the diagnostic performance of our RAA method in clinical circumstances, 135 clinical urine samples ordered for urine culture were included. Urine culture was performed following standard protocols. Briefly, the urine samples were inoculated onto both Columbia blood agar plate and China Blue agar plate with a 10 μL quantitative inoculation loop. Then the plates were incubated in 5% CO_2, 35℃ for 48 hours. Observations were made every 24 hours of incubation. If pure culture or ≤ 2 species of bacteria grow on the medium and bacterial counts ≥ 5 x 10^3 CFU/mL, the urine culture result is considered positive. Then Matrix-assisted Laser Desorption/ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) was performed for bacterial identification. The urine culture negative results were reported if no bacteria growth was seen in plates at 48 hours.

RAA assay was performed in parallel with the remaining samples, as described in this study. Sensitivity and specificity were calculated using the formula of "(True Positive)/(True Positive + False Negative)" and "(True Negative)/(True Negative + False Positive)", respectively. Accuracy was calculated using the formula of "(True Positive + True Negative)/(True Positive + True Negative + False Positive + False Negative)".

RESULTS

Determination of primer combination

Amplification was performed using different probe and primer combinations, and amplification curves were compared (Figure 2). The primer 1F+2R combination showed the strongest amplification signal; therefore, the primer combination 1F+2R was optimal and used for subsequent assays. The sequences of 1F+2R primers and probe were shown in Table 1.

Specificity assay

Only *E. coli* ATCC35218 was positive in the RAA assay, and other uropathogens were negative (Figure 2). Thus, our RAA method did not cross-react with other common uropathogens, showing good specificity.

Determination of LOD

The amplification curve showed that the fluorescence intensity of different concentrations of template decreased gradually with serial dilution. We enriched pathogens by centrifuging 10 mL of urine followed by addition of 100 μL deionized water. Thus, after this calculation, the serial dilution concentrations were shown in Figure 4. Clear and reproducible amplification signals were still detectable when the DNA concentration was reduced to 10^3 CFU/mL. However, the amplification signal disappeared when the DNA concentration reduced to 10^2 CFU/mL. So, the minimal detectable DNA concentration was 10^3 CFU/mL, which was determined to be LOD of this RAA assay.

Diagnostic performance of RAA compared with urine culture

Among the 135 urine culture results, 50 of them were *E. coli.,* 75 of them were non-*E. coli*. The remaining 10 were culture negative. The detailed results of RAA compared with urine culture were shown in Table 2. The 10 urine culture negative samples were all RAA negative, with 100% agreement. Three samples were RAA negative and urine culture positive, and 7 samples were RAA positive and urine culture negative. These discrepant samples were retested, and they were the same as initial results. The sensitivity, specificity and accuracy of RAA assay compared with urine culture were 94.00%, 91.76%, and 92.59%, respectively.

DISCUSSION

Urine culture is still the gold standard to determine UTI pathogens, but it usually takes 2 - 4 days to get the results. Thus, it cannot guide initial antibiotic therapy, which usually ends up being empirical and potentially inappropriate [15]. Rapid identification of UTI pathogens for initial antibiotic therapy could contribute greatly to lower the clinical and economic burden of UTIs. Molecular methods such as real-time PCR, Loop-Mediated Isothermal Amplification (LAMP), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), have reportedly been used for detecting various pathogens in different kinds of samples. Real-time PCR method successfully detected the first SARS-CoV-2, reflecting the power and usefulness of this technique [16]. Real-time PCR has high sensitivity and specificity. However, it demands specialized PCR laboratory equipment, sophisticated expertise, and expensive machines. LAMP amplifies DNA with high specificity, efficiency, and rapidity. However, the designing and screening of primer pairs are sophisticated and it is more prone to

Table 1. Primers and probe sequences.

Primers/ probe	Sequence 5'-3'	Gene	Positions
1F	AACTAACGAAACTCAGGAGTACGGTT	*chuA*	1,894 - 1,919
1R	TATATTCCCCAGTATCGGTGTCTTTGCC	*chuA*	2,145 - 2,172
2F	GATGACCTGATGTTGTCCAATGATGCT	*chuA*	1,934 - 1,960
2R	TACCAACCCACCCAACAGAGAAGCCACT	*chuA*	2,229 - 2,256
3F	CAAAGCGAAGGATTACATCTCCACGACCG	*chuA*	1,990 - 2,018
3R	GCTGTAACTGCTGCTGATATGTGTTGAGC	*chuA*	2,268 - 2,297
Probe	ACCACTGATCTGTTTAGCCTTGATGTGGCC(FAM-dT)A(THF)[BHQ-dT]AACCGTACCCGCGGC-C3 Spacer	*chuA*	2,099 - 2,147

F - forward primer, R - reverse primer, FAM-dT and BHQ1-dT - two conserved thymine remnants (dT) within the probe, THF - tetra-hydrofuran.

Table 2. Comparison of RAA results with urine culture.

	Urine culture for *E. coli*	Urine culture for non-*E. coli* and negative	Total
RAA positive	47	7	54
RAA negative	3	78	81
Total	50	85	135

Figure 1. Position of primers and probe on *chuA* gene.

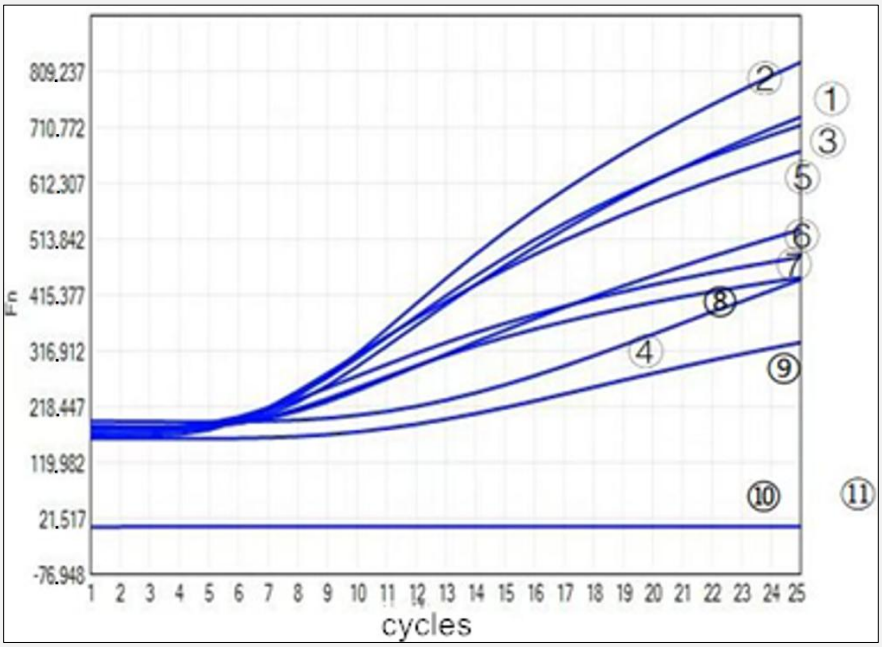

Figure 2. Amplification plot of different primers and probe combinations.

(1) 1F+1R, (2) 1F+2R, (3) 1F+3R, (4) 2F+1R, (5) 2F+2R, (6) 2F+3R, (7) 3F+1R, (8) 3F+2R, (9) 3F+3R, (10) Negative control (no primers), (11) Negative control (no template,1F+1R).

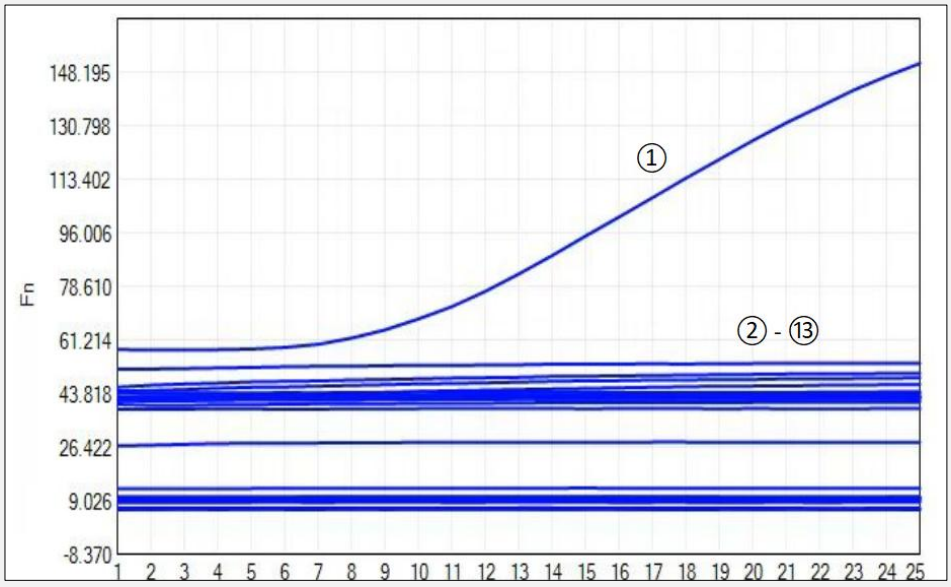

Figure 3. Amplification plot of specificity assay.

(1) - *E. coli*, (2) - (13) - none - *E. coli.*

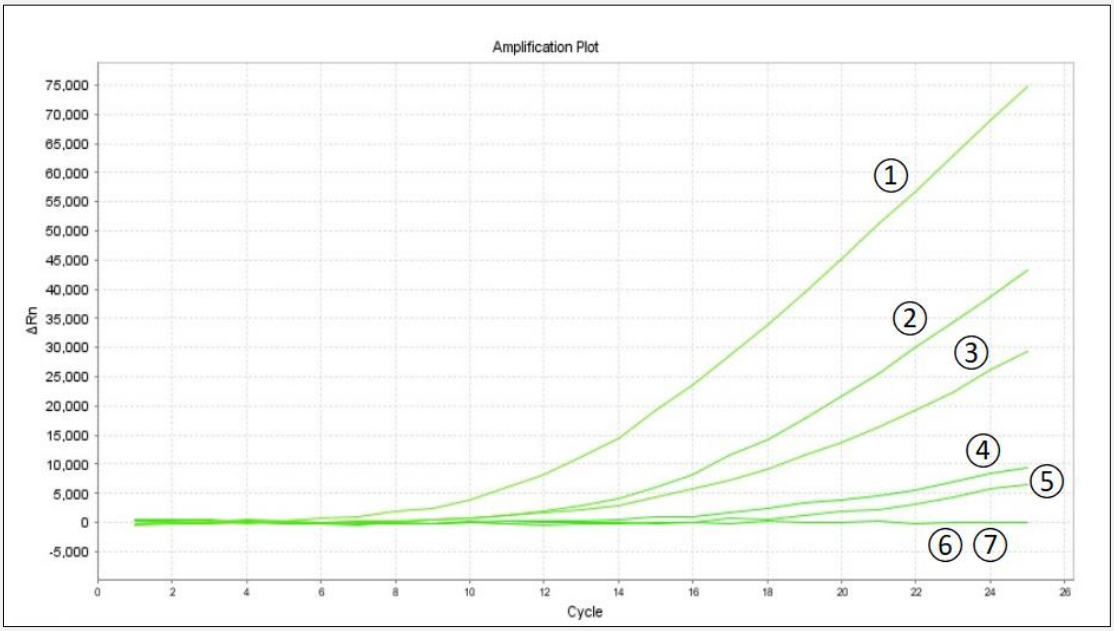

Figure 4. LOD of RAA method for *E. coli.*

The calculated LOD (due to the 100 times enrichment of original urine samples) were as follows: ① 10^7 CFU/mL, ② 10^6 CFU/mL, ③ 10^5 CFU/mL, ④ 10^4 CFU/mL, ⑤ 10^3 CFU/mL, ⑥ 10^2 CFU/mL, ⑦ 10 CFU/mL.

contamination which leads to false positive results [17]. CRISPR is a novel method developed in recent years. Cas proteins can recognize and support cis-cleavage of target DNA or RNA, then activate Cas proteins to indiscriminately cleave non-targeted single-stranded DNA. Thus, it could be used to detect pathogens' nucleic acids [18]. However, Cas proteins are expensive. CRISPR has high specificity, but its sensitivity is low. It is often used in combination with PCR or other isothermal amplification methods [19].

RAA originated from recombinase polymerase amplification (RPA), which is a promising and emerging technology for rapidly amplifying target nucleic acid from minimally processed samples [20]. Compared with the above-mentioned molecular methods, RAA has the advantage of high sensitivity and specificity. It is low-cost, does not require sophisticated reading tools, and is simple to use. Thus, it is suitable for POCT and on-site field testing [21]. Moreover, we used fluorescence probe RAA method to monitor the real-time amplification plot without opening the lid. This fluorescence RAA method is better than using chromogenic stripe for observing results. It could significantly reduce amplification products contamination. In this study, we developed an RAA assay for rapidly detecting urinary *E. coli* and evaluated its clinical utility compared with urine culture. It could provide detection results within 30 minutes. To the best of our knowledge, we are the first to develope the RAA assay specifically targeting the UTI main pathogen *E. coli*.

In our study, 135 clinical urine samples were included to evaluate the RAA assay, with the sensitivity, specificity, and accuracy of 94.00%, 91.76%, and 92.59%, respectively. It indicated the RAA assay established in this study is in well-consistency with the results of urine culture. A previous study evaluated RAA in detecting Methicillin-resistant *Staphylococcus aureus* (MRSA) from dairy farms, compared with Taqman probe qPCR assay, with the overall agreement of 97.01% [22]. Real-time fluorescent RAA could also detect HBV with the sensitivity and specificity of 97.18% and 100%, respectively [23]. They showed RAA had good performance in different types of specimens.

In our study, 3 samples with positive urine culture results of *E. coli* were RAA negative. This was probably due to the complex and inhibitory components of these urine samples, e.g., the patients were elderly with long-term usage of multiple kinds of medications. Another 7 samples with RAA positive results were urine culture negative. This was probably due to the fact that LOD of RAA is 10^3 cfu/mL, while the bacterial count threshold of urine culture is 5 x 10^3 cfu/mL following the Stan-

dard Operation Protocol (SOP) of our laboratory. Urine bacterial count < 5 x 10^3 cfu/mL is considered contamination and reported urine culture negative. Thus, this was probably the reason of these 7 RAA positive but urine culture negative discrepant results.

In conclusion, we have successfully established an RAA assay based on a conserved fragment of *E. coli chuA* gene. It has good sensitivity, specificity, and accuracy compared with urine culture. It is a rapid, low cost, and reliable method for preliminary screening of *E. coli* in UTI. Furthermore, our RAA method could also extend to detect other uropathogens with different pathogen-specific primer and probe combinations in future.

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Declaration of Interest:

The authors declare no competing financial interests and declare no generative AI with scientific writing was used.

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