Simultaneous Detection of Chlamydia Trachomatis, Neisseria Gonorrhoeae, Ureaplasma Urealyticum by Multiplex PCR-Running

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Abstract:Chlamydia trachomatis (CT), Ureaplasma urealyticum (UU) and Neisseria gonorrhoeae (NG) are the most common pathogens of sexually transmitted infections (STIs), frequently founded in urogenital infections, and showed a criminal role in increasing the risk of potential adverse outcomes. In this study a multiplex PCR assay for the simultaneous detection and accurate identification of 3 clinically relevant pathogens of STIs, i.e., CT, NG and UU in a single tube was developed and evaluated. The limits of detection for the multiplex PCR assay were ~10 copies of DNAs per reaction. This assay has comparable clinical sensitivity to the conventional monoplex real-time PCR assay and considerable potential to be routine molecular diagnostic tool for simultaneous identification of STIs at relatively low cost due to multiplexing.

Keywords: Sexually Transmitted Infection; Multiplex PCR; Chlamydia Trachomatis; Ureaplasma Urealyticum; Neisseria Gonorrhoeae

Introduction

Chlamydia trachomatis (CT), Ureaplasma urealyticum (UU) and Neisseria gonorrhoeae (NG) as the most frequent pathogens of sexually transmitted infections (STIs) are considered to be the main etiological agents of urogenital disease (Xu et al. 2018; Roy et al. 2021). Genital sexually transmitted CT, UU and NG are highly prevalent in women (Liang et al. 2018), increased the risk of potential adverse pregnancy outcomes, such as preterm birth, low birth weight, spontaneous abortion, perinatal mortality and ophthalmia neonatorum (Vallely et al. 2018), newborn respiratory distress syndrome.

To avoid the abuse of antibiotic, guidelines for sexually transmitted infected urethritis suggested that infected with high load should be treated rather than routine and treatment of asymptomatic person (Horner et al.2018). Therefore, the accurate determination of pathogens can avoid the risk of antibiotic resistance and overuse of antibiotics indicated for empirical treatments (Bartoletti et al. 2019).

Nucleic acid amplification tests performed more specific and sensitive than culture or enzyme immunoassay tests (Marangoni et al. 2012) and increased the finding of case (Pillay et al. 2021). Molecular diagnosis by PCR directly detects pathogen-specific nucleic acid, many of the recently diagnostic methods for STIs employ PCR methods (Muvunyi et al, 2011;Van Der Pol et al. 2017). Several commercial multiplex PCR kits have been developed and evaluated (Ursi et al. 2016; Barrientos-Durán et al. 2020).

In this survey, multiplex real-time PCR was optimized for accurate identification of CT, UU and NG in a single PCR tube with the use of three fluorescent probes.

1. Materials and Methods

1.1 Clinical specimens

The DNA samples from the patients were obtained from Hang Zhou KingMed Diagnostics Co., Ltd. Briefly, the swabs was placed in a sterile test tube containing 1 mL of sterile normal saline as the transport medium and washed by shock for a while. The swab was then squeezed dry on the wall and discarded. Then, the samples were stored at -20°C for further treatment.

1.2 Primer and probe design

According to the genome sequences of CT, UU and NG from GeneBank, the software Primer Express was used to design PCR primers and their corresponding TaqMan MGB probes. Melting temperature (Tm) values and secondary structures were mainly considered. All primers were desalted or HPLC purified grade.

1.3 PCR assay design

The PCR Amplification reactions were accomplished in a final volume of 25 μL. The PCR products were run on 2% agarose gel electrophoresis. Real-time PCR was conducted using the ABI7000/7300/7500 Real-Time PCR System and LightCycler 2.0.

1.4 PCR sensitivity and specificity

Using the optimized PCR conditions, the sensitivity and specificity of monoplex and multiple PCRs were analyzed with clinical samples and plasmid standard DNAs, respectively. A 10-fold diluted DNA series of 10^5, 10^4, 10^3, 10^2, 10 copies per reaction were used to assess sensitivity levels. PCR cycle conditions: 50 ℃ 2 min, 94 ℃ 2 min, 95 ℃ 15 s, 60 ℃ 45 s; 45 cycles. 11 pathogen DNAs of Actinomyces isrealii, Aerococcus viridians, Enterococcus faecalis, Enterobacter cloacae, Morganella morganii, Mycoplasma hominis, Pseudomonas aeruginosa, Streptococcus epidermidis, Candida albicans, Herpes simplex virus 1 and Herpes simplex virus 2 with urinary tract infection were selected to confirm the specificity.

2. Results and Discussion

2.1 Primer and probe screening

In this study, the specificity of primers was screened by using human whole genome DNA and other pathogens' nucleic acid as PCR template. Based on the results of specific amplification and sequencing, primer pairs of C1f/C1r; N1f/N1r; U5f/U5r were used for multiple PCR as shown in **Figure 1**. According to the screened primer sequence, the optimized primers and probes' sequences in **Table 1** were finally selected for multiplex real-time PCR system.

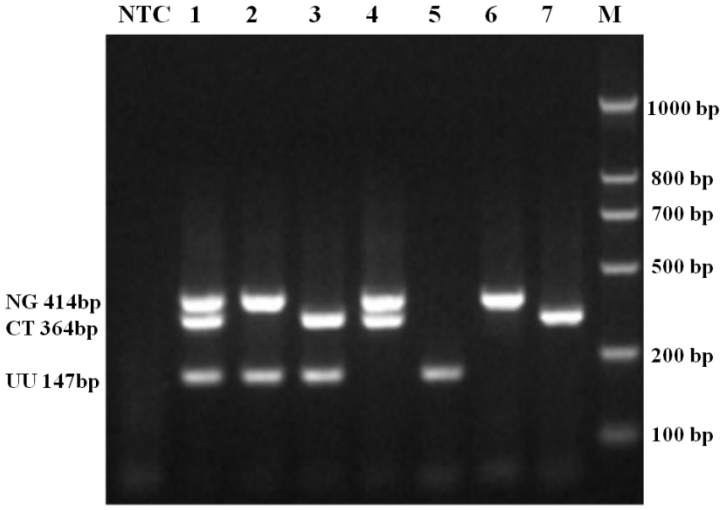

Figure 1 Multiple PCR analysis of mixed 3 primer pairs. CT/NG/UU (lane 1), NG/UU (lane 2), CT/UU (lane 3), NG/CT (lane 4), UU DNA (lane 5), NG DNA (lane 6), CT DNA (lane 7).

Table 1 Optimized primers and probes

Assay	Primer Sequence (5'→3')	Probe Sequence (5'→3')	Product size
CT	C1f (27-mer): GCAAGATATCGAGTATGCGTTGTTAGG C1r (25-mer): TTCATTGTACTCATTAAACGAGCGG	C1 probe (TaqMan MGB probe, 18-mer): VIC-AAAGATATGGACAAATCG-MGB	364 bp
NG	G1f (21-mer): TATCGGAACGTACCGGGTAGC G5r (21-mer): GTATTACCGCGGCTGCTGGCA	N1 probe (TaqMan MGB probe, 16-mer): NED-CCGATGACGGTACCTG-MGB	414 bp
UU	U5f1 (26-mer): GTCAGGATCATCAAATCAATTCACAC U5r1 (23mer): GATCCAACTTGGATAGGACGGTC	U2 probe (TaqMan MGBprobe, 17-mer): FAM-CCAGGAGCAATTAACT-MGB	147bp

2.2 Multiplex real-time PCR assay

Simultaneous amplification and detection of the three pathogens were achieved using the screened primers and fluorescent probes listed in **Table 1** and verified by electrophoresis (**Figure 1**). In the presence of a single target, the multiplex PCR produced detection limits of 10 copies of CT, NG or UU plasmid control DNAs, respectively, whereas detection limits were unchanged when two targets (CT/NG, CT/UU, or UU/NG) or all three targets were present in the PCR mixture.

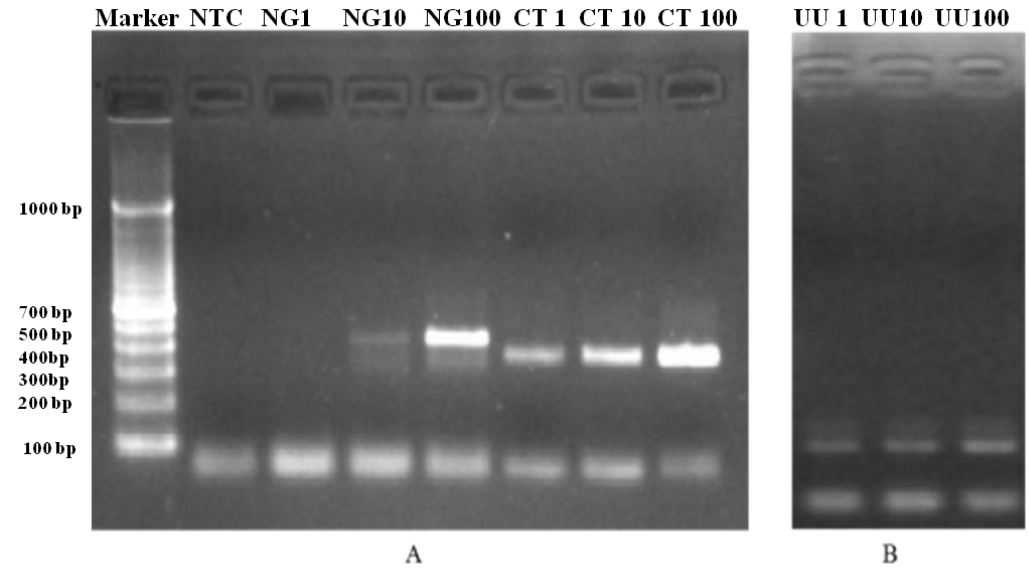

Figure 2 Evaluation of the limit of multiplex PCR. DNA copy number gradients for all three pathogens were 1 copy, 10 copies and 100 copies per reaction.

Probe-based multiplex real-time PCR could simultaneously distinguish multiple pathogens (Carrillo *et al*. 2020). Here, new probes as shown in **Table 1** were designed and individually examined with crude DNAs of CT, NG and UU (data not shown) by the multiplex real-time PCR. The developed multiplex real time PCR of high throughput was successfully established in this survey and can be used as a simple and useful alternative to the monoplex real-time PCR for rapid and accurate identification of CT, NG and UU.

3. Acknowledgements

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4. Conflict of interest

The authors declare that they have no conflicts of interest.

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