Letter to the Editor

Establishment of Multiplex PCR for Simultaneous Detection of Four Venereal Pathogens*

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Venereal diseases are considered to be the most prevalent infectious diseases in the worldwide. China is now faced with a year-by-year increasing incidence of sexually transmitted diseases (STD), which are spreading from high-risk groups to the general population. *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum* and herpes simplex virus-2 (HSV-2) are always regarded as the most common venereal pathogens. The "golden standard" for testing *Neisseria gonorrhoeae* remains to be bacteria culture or microscopic examination. The sensitivity of these testing methods, however, is seriously influenced by some factors. High-quality specimen as well as fast inspection is required considering the poor viability of *Neisseria gonorrhoeae in vitro*. Furthermore, misuse and overuse of antibiotics can also contribute to atypical symptoms which also reduce the sensitivity of conventional methods mentioned above. Thus, common PCR and fluorogenic quantitative PCR have played the role of routine screening methods[1-2]. *Chlamydia trachomatis* and *Ureaplasma urealyticum* are prone to be inactive *in vitro*. For detection of *Chlamydia trachomatis* and *Ureaplasma urealyticum* with conventional biocytoculture is time-consuming and has high requirements on technology and equipment, it has been gradually replaced by new detecting techniques including rapid immune chromatography based on the molecular biology, direct fluorescent or gold-immunochromatographic assay, enzyme immunoassay, PCR techniques and etc.. Traditional laboratory testing for HSV infection depends on HSV isolation and culture as well as immunological methods, which is also time-consuming and low sensitive. Then PCR techniques and micro cantilever immune sensor gradually came into use[3-5]. People who are infected with more than one sexually transmitted disease simultaneously are called mixed infectors, making up a high proportion of STD patients[6-7]. Establishing methods to detect several venereal pathogens at the same time, therefore, is of great practical significance to improve clinical diagnostic efficiency. It is difficult to apply conventional diagnostic methods based on pathogen separation to screening of mixed infection with multiple venereal diseases. Multiplex PCR could provide the convenience of detecting various pathogens by once running, with great advantages to save time and cost. This technique is increasingly used in diagnosis of various diseases[8-9], while only McKechnie reported a method where four venereal pathogens were simultaneously detected by a multiplex PCR-Based reverse line blot assay in urine[10]. Thus, it is useful to develop a multiplex PCR technique, which could provide a fast detection method to simultaneously detect four pathogens, including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum* and herpes simplex virus-2.

Conservative genes were chosen as target genes and suitable primers were designed to detect pathogens, which served as the foundation of PCR techniques. Currently, specific genes available for clinical detection of *Neisseria gonorrhoeae* are cytosine DNA methyltransferase (COMT), 16S ribosomal rRNA, Cryptic Plasmid DNA, ORF1, Opa and Por, which all manifest higher sensitivity than bacterial culture and smear microscopy inspection and the specificity higher than 95%. Target genes for PCR detection of *Chlamydia trachomatis* include the major outer membrane protein, cryptic plasmid and 16S rRNA genes. Target genes for PCR detection of *Ureaplasma urealyticum* include multiple-banded antigen (MBA) gene, 16S rRNA gene and urease gene. As HSV-1 and HSV-2 can infect humans with 40% homology, they need to be identified when PCR detection is conducted. Such information provided reference for primers design in this study. The following general requirements were also considered: Various primers have similar T_m values; primers will

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not form a dimer; target fragment gap should exceed 30bp to meet requirements on the resolution in agarose gel electrophoresis. Then, four pairs of primers were devised based on target sequences of *Neisseria gonorrhoeae* strain B-340 porin IB protein gene, *Chlamydia trachomatis* A2497 gene, *Ureaplasma urealyticum* urease gene, human herpes virus 2 isolate 06490396 DNA polymerase (UL30) gene by Primer Premier 5.0 software, as shown in Table 1.

Table 1. Multiplex PCR Primers and the Corresponding Information of Targets Genes

Pathogens	Primers (5'-3')	Amplicon Size (bp)	Gene No.
Neisseria gonorrhoeae	F: GATGTCACCCTGTACGGTGCCATCAAA R: GGATTCCCAAGCATTGACGTT	327	GU058271.1
Chlamydia trachomatis	F: GTAGCCCTACCGGAAGGTGG R: CAAACTATATGTCTCGTCCTCACC	167	CP002401.1
Ureaplasma urealyticum	F: TCTGCTCGTGAAGTATTAC R: ACGACGTCCATAAGCAAC	426	AF085729.2
Herpes simplex virus 2	F: ATGGTGAACATCGACATGTACGG R: CCTCCTTGTCGAGGCCCCGAAAC	391	HQ123177.1

To obtain the target PCR template, four venereal pathogens including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum* and herpes simplex virus-2 were obtained from body fluid of respective carriers with each diagnosed as being infected with only one sexually transmitted disease and cultivated. Then, genomic DNA of these four venereal pathogens was separately extracted by using DNA extract kit (Daangene, China).

After primers and templates were prepared, method development was performed, which mainly consisted of the following four steps. Firstly, a single PCR amplification for four pathogen genomes was conducted. Results showed that each pair of primers only reacted with the respective substrate template and the specificity and sensitivity of each pair were satisfactory. After DNA electrophoresis, expected length of the strip only existed in the corresponding position without miscellaneous bands and the target bands were confirmed after being sequenced. Secondly, the multiplex PCR system was optimized by using multiplex PCR Assay kit (TaKaRa, Dalian): Multiplex PCR Mix 10.25 μL, Multiplex PCR Mix 225 μL, pathogen DNA template 5 μL, each primer (0.2 μmol/L) 0.5 μL, dH_2O 15.75 μL. The reaction conditions were: initial 94 °C for 5 min; then 35 cycles at 94 °C for 30 s, 60 °C for 90 s and 72 °C for 90 s; and final 72 °C for 10 min. PCR products were confirmed by agarose gel electrophoresis. Thirdly, the specificity of this method was tested by amplifying with various pathogens DNA as templates under the optimized multiplex PCR system. The results showed that target bands appeared when single pathogen (including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum*, herpes simplex virus-2) genomic DNA as well as four mixed pathogen genomic DNA was taken as templates. Nevertheless, no any fragment was amplified from other non-purpose pathogen DNA, as shown in Figure 1. Finally, the method was preliminary validated by testing 82 real samples, all were collected from urethral discharge and pus etc. of patients with typical symptoms. Positive results with 19 samples of *Neisseria gonorrhoeae,* 18 samples of herpes simplex virus -2, 24 samples of *Chlamydia trachomatis* and 22 samples of *Ureaplasma urealyticum* were obtained under optimized multiplex PCR reaction conditions. The results were also accord with the clinical diagnosis, except that one sample diagnosed as *Chlamydia trachomatis* was tested by multiplex-PCR to be positive in both *Chlamydia trachomatis* and *Ureaplasma urealyticum*. This sample was finally confirmed by routine clinical culture tests as infected with these two venereal pathogens. These results show great consistency to those of clinical routine examinations.

The study indicates that the constructed multiplex PCR can provide a reliable tool for diagnosis of mixed infected venereal diseases, with important application value for simultaneous, specific and rapid screening of four common sexually transmitted pathogens.

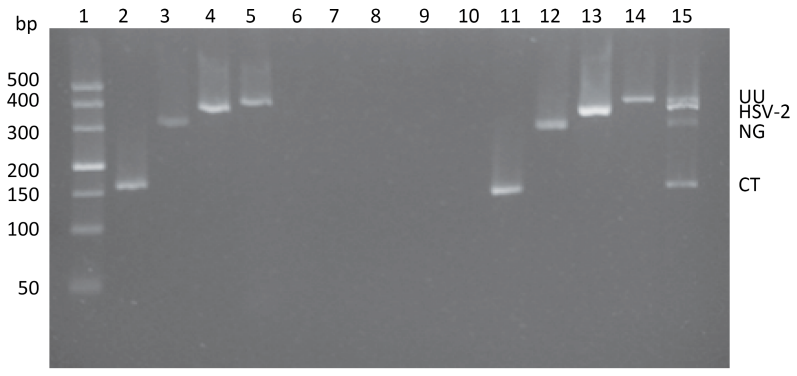

Figure 1. 1: DNA marker: The figure shows 50, 100, 150, 200, 300, 400, and 500 bp respectively in a bottom-up manner; 2-5: Single fragment was amplified from single primer corresponding to single genome of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, herpes simplex virus-2 and *Ureaplasma urealyticum*. 6-10: Genomes of *Gardnerella vaginalis*, *Trichomonas vaginalis*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, colon *Aye bacterial* were amplified with mixture of four groups of primers; 11-14: Genomes of *Chlamydia trachomatis*(CT), *Neisseria gonorrhoeae* (NG), herpes simplex virus -2 (HSV-2) and Ureaplasma urealyticum (UU) were respectively amplified with four groups of mixed-primers by using multiplex PCR; 15: Products of mixture of four target genomes were amplified from four groups of mixed-primers by using multiplex PCR.

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