

## Development of Multiplex-PCR Assay for the Rapid Diagnosis of Four Prevalent Sexually Transmitted Agents

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### Abstract

**Objectives:** Sexually transmitted diseases (STDs) is a globally major health concern and with respect to WHO, about 340 million new infection occur around the world, annually. STDs pathogens, has ability to cause severe complication in patients, like pelvic inflammatory diseases (PIDs), ectopic pregnancy, genital cancers as well as neonatal morbidity and mortality. Because of conventional methods limitations for detection of STDs pathogens, this study aim to develop multiplex PCR (M-PCR) as a cost effective, rapid and highly sensitive and specific for simultaneous detection of mostly frequent STDs agents follow as: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and group B streptococcus.

**Methods:** In this investigation, endocervix secretions specimen were collected from 100 no pregnant women who were doubtful for STDs and referred to Kamali hospital. DNA extraction was performed on patients sample and standard strain of each mentioned STDs pathogen. Conventional PCR amplification was down on standard strains. Regard to PCR result, best melting temperature selected and M-PCR performed on DNA template of the standard strains and patients sample.

**Results:** Prevalence of *C. trachomatis*, *M. genitalium*, *N. gonorrhoea* and *S. agalactiae* infection was found to be 16%, 5%, 9% and 15% out of 100 patients respectively. M-PCR validation parameters were calculated and observed to be excellent including sensitivity (100%) and specificity (range between 96.5 - 98.9%).

**Conclusions:** With regard to high prevalence rate for STDs in Iran, we suggested that M-PCR should be consider as a part of the national health screening programs to reduce the burden of disease on patients and government.

**Keyword:** Multiplex PCR; Sexually Transmitted Diseases

### Introduction

STDs is an extremely health concern around the world and create major medical, social and economic problems in communities and consider as an important disease in term of morbidity and mortality in sexually active individuals [1]. STDs has worldwide distribution and according to WHO, more than 340 million new cases occur all over the world, annually, with highest rate in developed communi-

ties [2]. *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and group *B. streptococcus* (*Streptococcus agalactiae*) are known as important STDs agents [3].

STDs agents, specially *Neisseria gonorrhoea* and *Chlamydia trachomatis* can cause PIDs as well as infertility in both men and women. Beside, other complication, such as ectopic pregnancy, genital cancers, neonatal morbidity and mortality and chronic pelvic pain have been associated with STDs [1,2].

Sexually transmitted infections, can be transmitted from mother to child and lead to stillbirth, neonatal death, low-birth-weight and prematurity, sepsis, pneumonia, neonatal conjunctivitis, and congenital deformities [1]. Most of sexually transmitted infections are asymptomatic or has slight symptoms, that make diagnosis process difficult [1]. Several methods exist for detection of STDs pathogens, including: culture method, biochemical test, direct microscopic slide, Enzyme Linked Immuno Sorbent Assay (ELISA), monoplex and multiplex PCR and ligase chain reaction [4-10]. Among those, PCR method represented as a highly sensitive and specific diagnostic way. In addition, PCR provides a rapid way for detection within a day [11,12]. Multiplex PCR assay has an additional advantage, because of ability to simultaneous detection of multiple pathogens.

In this investigation we developed a Multiplex-PCR assay for detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and group *B. streptococcus* as most common and frequent STDs pathogens in Iran.

### Material and Method

#### Location of study and sample preparation

This study was carried out at cellular and molecular laboratory of Alborz University of Medical Sciences, located at Alborz province, Iran.

Standard strain of *Chlamydia trachomatis* (D and L2 serovar) (Guangming Zhong, M.D., Ph.D. Department of Microbiology and Immunology, Texas Health Science Center University, San Antonio), *Neisseria gonorrhoeae* (N239) (Avicenna Research Institute, Tehran, Iran), *Mycoplasma genitalium* G<sub>37</sub> (Sabine Pereyre University of Bordeaux, Burdeos, Aquitaine, France) and group *B. streptococcus* (ATCC13813) (PTCC - Persian Type Culture Collection) provided. In this investigation, 100 pregnant women who referred to Kamali hospital (located in Alborz, Iran) and were doubtful for STDs were included. In addition, ethical approval and patient consent were obtained. (Ethics committee approval number: ABZUMS.REC.1394.13)

Endocervix secretions specimen aseptically were collected from patients by using sterile swab regard to standard protocol. Consequently, swab put into standard 2SP media (Technical Data #2447KR / 2013.06.05) and transported to molecular laboratory of Alborz University, immediately, then stored at -20°C until subsequent use.

#### Genomic DNA extraction

Genomic DNA of each standard species and patient's specimen was extracted using commercial DNA extraction kit (QIAamp DNA mini kit, Qiagen Inc. GMBH, Germany) according to manufacturer instruction.

Briefly, 1 ml of broth bacterial culture added to 1.5 ml microcentrifuge tube and then centrifuged at 7500 RPM for 5 minutes. After discarding the supernatant, ATL buffer added with final volume of 180 µl, then 20 µl of proteinase K was added. The tube was vortexed for 15 second and incubated at 56°C for 3 hours. AL buffer added to mixture then incubated for 10 minutes at 70°C. 200 µl of absolute ethanol add and mix thoroughly by vortexing the tube. After brief centrifuge, supernatant applied to the QIAamp spin column and centrifuged at 8000 rpm for 60 second and flow-through was discarded. AW1 buffer added and centrifuged at 8000 for one minutes and flow-through

was discarded again. AW2 buffer add and centrifuge at 14000 for 1 min and transferred to the tube new 2 ml microcentrifuge tube. AE buffer was added and stand for 1 min at room temperature and it was centrifuged at 8000 rpm for one minutes. DNA extraction product evaluated using NanoDrop (Thermo Scientific 1000) and then was stored at -20°C until use.

## Polymerase Chain Reaction

### Primer design

Sequences of each proposed target gene obtained from gene bank and then the primers with compatible melting temperatures designed against highly conserved region for each pathogen using Allele ID Software version 6 (Table 1). Subsequently, primers were evaluated using BLAST analysis in the NCBI database.

Organism	Primer Sequence 5' → 3'	Target gene	Amplicon size (bp)
<i>C. trachomatis</i>	F-TAATGGTCACTCCTCGTAAG R-CCTCCTAAAGAATCTGAATGG	<i>MOMP</i>	845
<i>M. genitalium</i>	F-GGTATGATAACAACGGTAGAG R-GTAGTTCGCCTTGATTGAC	<i>MgPa</i>	474
<i>N. gonorrhoea</i>	F-ACGCCTGCTACTTTCAC R-CGGTATCACTCGCTCTG	<i>porA</i>	205
<i>S. agalactiae</i>	F-GTGTCTGCTGATAGTCCTG R-ATTCGTTCCGGTATTGATATGG	<i>cfb</i>	103

**Table 1:** Primer sequence.

### Monoplex PCR

Gradient PCR was performed for each standard strains in final volume of 25 µl. The PCR master mix was contain: distilled water (DW): 20 µl, each forward and reverse primer: 0.25 µl (10 pmol), dNTPs: 0.3 µl (10 mM), MgCl<sub>2</sub>: 0.5µl (50 mM), Buffer(10x): 2.5 µl, Taq DNA polymerase: 0.2 µl (500U), Template DNA: 1 µl (80-100 ng).

PCR performed by Eppendorf thermal cycler using followed thermal-cycling program: Initial denaturation at 94°C for 4 min followed by 30 - 35 cycle of 94°C for 1 min, gradient temperature for 1 min, 72°C for 1 min with final extension of 72°C for 12 min. At the end of amplification, 5 µl of PCR product load into 1% Agarose gel for 45 min and subsequently stained by Ethidium Bromide and visualized using an UV transilluminator.

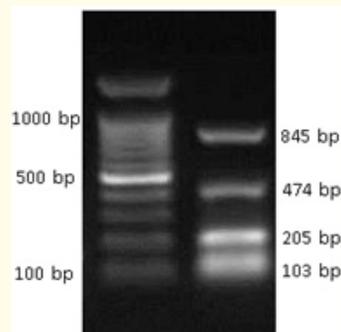
### Multiplex PCR (M-PCR) development

Regard to monoplex PCR result, compatible melting temperature were chosen for multiplex reaction and M-PCR was performed on the Standard species DNA and the Patient's DNA template in final volume of 50 µl contains: 28.8 µl DW, 0.5 µ of each primer (10 pmol), 0.6 µl dNTP (10 pmol), 1µl MgCl<sub>2</sub> (50 mM), 5 µl of PCR Buffer (10x), 0.6µl Taq DNA polymerase (500 U), 10 µl of template DNA (80 - 100 ng).

Cycling program was initial denaturation at 94°C for 4 min, followed by 30 cycle of 1min at 94°C, 1 min at 54.5°C, 1 min at 72°C. Final extension was 72°C for 12 min. PCR products were evaluated by agarose gel electrophoresis. Regard to the patients culture and PCR result, specificity and sensitivity was determined by this formula.

## Result

Gradient polymerase chain reaction performed on each standard strain DNA template and annealing temperature range of 48 - 63.8, 50 - 63.3, 51 - 63.1, 45 - 55.3 observed to be best range for *C. trachomatis*, *M. genitalium*, *N. gonorrhoea* and *S. agalactiae* respectively. Annealing temperature of 54°C was chosen as best temperature for multiplex reaction and consequently M-PCR performed at suitable PCR condition on standard strain and patient's specimen (Figure 1).



Prevalence of *C. trachomatis*, *M. genitalium*, *N. gonorrhoea* and *S. agalactiae* infection among 100 patients was found to be 16%, 5%, 9% and 15% respectively. Simultaneous infection by three pathogen detected in only one case while infection by four pathogen were not seen in none of the patients. According to the formula, specificity and sensitivity were calculated, shown in table 2.

Organism	Specificity	Sensitivity
<i>C. trachomatis</i>	98.8%	100%
<i>M. genitalium</i>	97.9%	100%
<i>N. gonorrhoea</i>	98.9%	100%
<i>S. agalactiae</i>	96.5%	100%

**Table 2:** Specificity and sensitivity.

## Discussion

In this investigation, we developed multiplex PCR for simultaneous detection of four frequent type of bacterial STDs pathogen including *C. trachomatis*, *M. genitalium*, *N. gonorrhoea* and *S. agalactiae* in endocervix secretion specimen for first time in Iran.

Consequently, we perform M-PCR on 100-endocervixsecretion specimen. Prevalence rate of *C. trachomatis*, *M. genitalium*, *N. gonorrhoea* and *S. agalactiae* infection was found to be 16%, 5%, 9% and 15% respectively among our patient.

The M-PCR method illustrated has many advantages in compare to conventional diagnostic methods. For instance, M-PCR is rapid way with high specificity and sensitivity for detection. Asymptomatic patient would be an important source of STDs and early diagnosis is most critical for control and limitation of STDs. This method allowed to early diagnosis of STDs, even in situation that patients have either no clinical manifestation or have mild and slight symptoms. Therefore, this method reduce cost and burden on patients and health care systems as well as avoid rising trend of STDs prevalence in the communities. Furthermore, applying M-PCR in clinical laboratories, provide saving time and money and reduce need to excess lab technicians.

The overall agreement of the M-PCR with single PCR was elevated (100%) and other validation parameters; including sensitivity and specificity were also excellent (ranging from 96.5% to 98.8%).

A study by Gimenes, *et al.* in 2014, detected seven sexually transmitted agents in semen by multiplex-PCR and found validation parameter such as specificity and sensitivity for M-PCR to be excellent (99.2% and 100% respectively) [13]. In another study by Ilami, *et al.* they detected *N. gonorrhoeae* and *C. trachomatis* in patients with symptomatic urethritis using multiplex PCR and the sensitivity was to be 99.3% which is in agreement with our finding [14]. Ahmadi, *et al.* in a meta-analysis, illustrated high prevalence of *C. trachomatis* in both men and women ranged from 0 to 23.3%, and 0 to 32.7% in different region of Iran respectively [15].

In conclusion, with respect to high prevalence of STDs in Iran, M-PCR can be considered as part of the national health screening programs, especially in high-risk groups to reduce the burden of disease on patients and government.

### Key Messages

- In this investigation M-PCR observed to be sensitive and specific diagnostic test.
- Prevalence rate for *C. trachomatis*, *M. genitalium*, *N. gonorrhoea* and *S. agalactiae* was high among our patients.
- M-PCR should be consider as a part of health screening programs.

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