Design multiplex PCR molecular technique to detect sexually transmitted agents, Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, herpes virus type 2, and papillomavirus

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ABSTRACT

Sexually transmitted infections (STIs) that cause sexually transmitted diseases (STDs) include various organisms such as bacteria, viruses, parasites, and fungi. These organisms are transmitted through sexual activity, which can increase problems such as infertility, ectopic pregnancy, and the risk of genital cancers. So quick diagnosis of sexually transmitted agents is important. In recent decades, the detection of microbial agents has been affected by using molecular techniques, because it is challenging and impossible to isolate a disease agent from clinical samples simultaneously and quickly. Most unsuccessful cases and time-consuming culture-based methods lead to the non-identification of microbial agents. This study aims to design a multiplex PCR technique for detecting sexually transmitted agents of *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, herpes virus type 2(HSV-2), and papillomavirus (HPV)* in 2022 in Qom, Iran. In the current study, about 100 Pap smear samples of patients in Qom City, Iran, were evaluated at a one-year time (in 2022)point for testing *HSV-2, HPV, Neisseria gonorrhea, Chlamydia trachomatis,* and *Trichomonas vaginalis* using multiplex PCR design. In the investigated samples, the frequency of *Chlamydia trachomatis, Neisseria gonorrhea, Trichomonas vaginalis, HSV-2, and HPV* was 8%, 5%, 3%, 12%, and 18%, respectively. HPV and *Chlamydia trachomatis* agents were found in 5 samples and HPV and *Trichomonas vaginalis* co-infection were observed in two samples. The PCR-multiplex method has higher speed, accuracy, specificity, and sensitivity. With this molecular technique, simultaneous infections can be detected faster and more accurately in clinical samples such as pubic smears, effectively speeding up treatment and reducing infection transmission.

Keywords: Sexually transmitted infections (STIs), sexually transmitted diseases (STDs), Molecular diagnosis

1. Introduction

The human urinary tract is a suitable place for the growth of microorganisms such as *Chlamydia trachomatis, Neisseria gonorrhea, Streptococcus agalactiae, Human papillomavirus (HPV), Mycoplasma genitalium, Ureaplasma urealyticum, Gardnerella vaginalis, Haemophilus dukrai, Mycoplasma hominis, Treponema pallidum, Ureaplasma parvum, Candida albicans, herpes simplex-1 (HSV-1) and herpes simplex-2 (HSV-2), HIV, HBV virus, and Trichomonas vaginalis (TV)* parasite. These microorganisms include bacteria, fungi, viruses, and

parasites, which are called sexually transmitted infections (STI) or Sexually Transmitted Infections. Globally, STIs are common among youth and adults. Organisms involved in sexually transmitted diseases (STDs) can cause infections in the human genitourinary tract, leading to infertility, pelvic inflammatory disease (PID), miscarriage, and inflammation. cervix in women and epididymitis, urethritis, and prostatitis in men (1,2,3).

According to reports, *Chlamydia trachomatis, Neisseria gonorrhea, Mycoplasma genitalium, Trichomonas vaginalis, Mycoplasma hominis, Uroplasma urealyticum, Uroplasma parvum, Herpes simplex virus (HSV)* are the most common STD pathogens. Some STD pathogens are treated with appropriate antibiotic therapy. However, most STD pathogens have unusual symptoms and are difficult to diagnose. Therefore, rapid and low-cost development of in vitro STD diagnostic screening methods will help to reduce STD-related genital damage and improve women's health worldwide (4,5,6).

Annual reports of the World Health Organization (WHO) indicate that approximately 340 million sexually transmitted diseases occur worldwide, with the highest rates in developing countries. However, according to WHO reports, STIs are more common in developed countries. Global statistics show that the prevalence of these infectious agents varies according to economic status, age, individual and community health, the number of sexual partners, and the social conditions of the community. Therefore, sexually transmitted diseases are generally considered a major global problem with devastating consequences, including financial loss and family damage (1,7,8). This study aimed to design a multiplex PCR molecular technique for the detection of sexually transmitted agents of *Neisseria gonorrhea, Chlamydia trachomatis, Trichomonas vaginalis, HSV-2, and HPV*.

2. Materials and Methods

2.1. Type of study and sample collection

In the present descriptive-cross-sectional study in 2022, first, sampling was done from patients who were referred to Qom, Iran for the presence of 2 *HSV, HPV, Neisseria gonorrhoeae, Chlamydia trachomatis,* and *Trichomonas vaginalis*. According to Cochran's formula to calculate the sample size, about 79 samples with a confidence factor of 1.96 should be tested, and 100 samples were tested in this study with a 10% probability of error.

$$n = \frac{Z^2_{1-\frac{\alpha}{2}}(P)(1-P)}{d^2}$$

2.2. *Design multiplex PCR molecular technique to detect Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, HSV-2,* and *HPV*

According to the instructions, DNA was extracted from the samples using the Cinna pure purification kit (Cinaclon Co, Iran).

Primers were designed using CLC Sequence Viewer and Gene Runner software and the NCBI website (Table 1). First, the target sequence for each of these factors was downloaded from the NCBI database using the CLC Sequence Viewer version 6 software, these sequences were placed under each other and designed based on the complete conserved regions of the primers. Finally, with the help of Gene Runner software version 6.5.52 beta, the thermodynamic properties of the primers were checked for each of the primers so that the secondary structure (primer dimer, loop, and hairpin structure) is not formed in the primers. For the multiplex reaction to be carried

out, it was tried that the reaction temperature for each of these agents should be in the same direction so that the reaction can be done in one run to detect these pathogenic agents.

Table 1. The sequences of the primers used

Microorganism	Target gene	Primer name	Primer sequence (5´-3´)	Tm (˚C)	Product (bp)
Chlamydia trachomatis	Phospholipase D Endonuclease Superfamily	F	TTTTAAACCTCCGGAACCC	51	
		R	GCATCGCATAGCATTCTTTG	51.8	347 bp
Neisseria gonorrhoeae	porA pseudogene	F	GTTGCGAATCCGTTTGGC	52	
		R	CGAAACCATGGGCATAGC	51.8	592 bp
Trichomonas vaginalis	Adhesive protein gene	F	CATGCCTTGTCCAGTTCGA	51	
		R	GCGGGAAACAGCCATATC	51	248 bp
HSV2	Glycoprotein D (US6) gene	F	CCTGCTAGTTGTCGCGGT	51	697 bp
		R	ATGCTGTCGACCGTCACG	51	
HPV	E6	F	CGTCCM*ARR*GGAW*ACTGATC	51	
		R	GCMCAGGGWCATAAY*AATGG	51	450 bp

*Degenerate primers are mixtures of similar primer sequences that incorporate variations at specific positions to account for the degeneracy of the genetic code *M:A ,C , R: A,G W:A, T ,Y: C,T .

To perform the PCR reaction, in the final volume of 25 µl, a mixture of 12.5 µl Mastermix (SinaClon Co, Iran), 1 µl of each of the primers and 5 µl of DNA template. The program for the thermocycler was optimized under the following conditions: Initial denaturation at 95 ° C for 2 min, 30 cycle of denaturation at 95° C for 30 sec, annealing at 51° C for 30 sec, extension of strands at 72 ° C for 40 sec, and final extension at 72 ° C for 5 min. Finally, 5 µl of each of the PCR products was poured into the wells of 2% electrophoresis gel and placed in the electrophoresis tank for 50 min and finally, the results were observed with the gel doc.

3. Results

During this research, about 100 samples of patients referred for HSV, HPV, *Neisseria gonorrhea*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* tests were prepared for medical diagnosis laboratories after receiving consent. After extracting the sample, qualitative and quantitative control of extraction was performed on the samples. Then, based on the primers designed for each PCR agent, and then after the set-up, a multiplex PCR test was performed. Based on this, in the M-PCR technique, for the samples of *Trichomonas vaginalis* band 248 bp,

chlamydia trachomatis with a product size of 347 bp, HPV with a product size of 450 bp, *Neisseria gonorrhea* with a product size of 592 bp and *HSV2* with The product size was observed to be 697 bp(Figure 1).

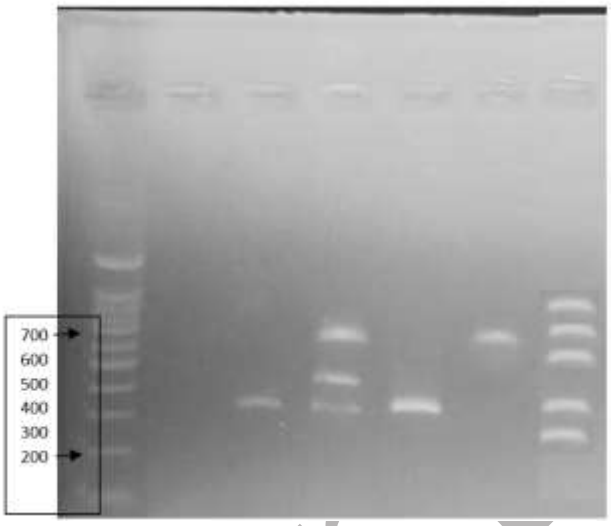

Figure 1. Multiplex PCR for samples of *Trichomonas vaginalis* 248 bp, *Chlamydia trachomatis* 347 bp, *HPV* 450 bp, *Neisseria gonorrhoeae* 592 bp, and *HSV2* 697 bp

3.1. The frequency of pathogenic agents in the samples

In this study, the frequency of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *HSV2*, and *HPV* in the available samples was 8%, 5%, 3%, 12%, and 18%, respectively (Figure 2). The samples were checked simultaneously with the molecular diagnosis kit of GA STD12 Plus RT-PCR KIT GeneovA company, Iran and the results were 100% consistent.

HPV and *Chlamydia trachomatis* were detected in 5 samples and *HPV* and *Trichomonas vaginalis* were detected in 2 samples.

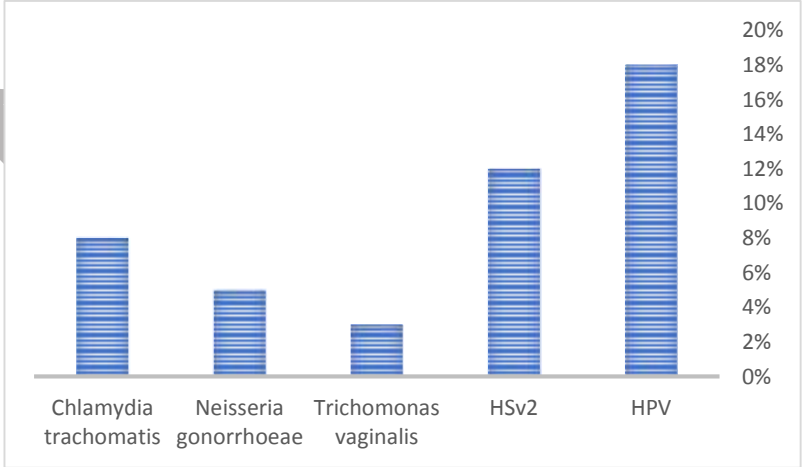

Figure 2. The frequency of pathogenic agents

4. Discussion

In this research, about 100 Pap smear samples were used to detect *HSV, HPV, Neisseria gonorrhoeae, Chlamydia trachomatis, and Trichomonas vaginalis* using the designed Multiplex PCR technique. In this study, the frequency of *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, HSV2, and HPV* was 8%, 5%, 3%, 12%, and 18% respectively. The samples were checked simultaneously with the molecular diagnosis kit of GeneovACompany, and the positive cases were confirmed with the kit based on Real-Time PCR. Also, the results showed that HPV and Chlamydia trachomatis agents were detected in 5 samples, and *HPV* and *Trichomonas vaginalis* infections were observed in 2 samples.

In the study by Amini et al, in 2021, was conducted on 60 infertile patients with symptomatic vaginal infection who were referred to Kerman Hospital, the frequency of infection with *Neisseria gonorrhoea* and *Toxoplasma gondii* was 6.6% and 10%, respectively. Co-infection with *Neisseria gonorrhoea* and *Toxoplasma gondii* was not detected in any of the samples(9). The results of this study showed that the multiplex PCR method was suitable for the diagnosis of *Neisseria gonorrhoeae* and *Toxoplasma gondii* in vaginal infection, which is consistent with the results obtained in the present study. In the study of Barrus et al, in 2016, an STI panel including several sets of PCR primers for each organism was designed for the detection of *Chlamydia trachomatis, Neisseria gonorrhoeae, Treponema pallidum, Trichomonas vaginalis, Mycoplasma genitalium, Ureaplasma urealyticum, Haemophilus ducreyi*, various types of *Haemophilus ducreyi* and *herpes viruses*. Among samples, 13% *Chlamydia trachomatis*, 7% *Neisseria gonorrhoeae*, 3% *Trichomonas vaginalis*, 2% HSV2, *Ureaplasma urealyticum* 12%, *Mycoplasma genitalium* 3%, and *Treponema pallidum* at 4%. The concordance between the Film Array STI panel and the standard nucleic acid amplification test was 98% for *Chlamydia trachomatis* and 97% for *Neisseria gonorrhoeae* (10).

Mahfouz et al, in their 2021 study performed a retrospective data analysis on all STD panels conducted at AUBMC from 2017 - 2019 to determine the molecular prevalence of eight different sexually transmitted organisms. Only 53.5% of samples were positive for one or more organisms. *Ureaplasma urealyticum/parum* was the most common pathogen 49.3%, followed by *Gardnerella vaginalis* 33.5%, *Chlamydia trachomatis* 5.36%, *Mycoplasma genitalium* 5.16%, *Neisseria gonorrhoeae* 2.5%, herpes virus 2.5%, and *Trichomonas vaginalis* 1.39%. In terms of pathogen distribution between genders, *Uroplasma urealyticum/parum, Herpes Simplex Virus (HSV),* and *Gardnerella vaginalis* were more common in women, and the rest were more common in men (11).

So, with the development of molecular techniques, STD screening with high sensitivity and specificity became easier. Yuan et al, in 2023 created a TP-HSV1-HSV2 multiplex polymerase chain reaction by targeting the conserved regions of the PolA TP gene and the UL42 gene of HSV1 and HSV2 to detect the skin lesions of 115 patients suspected of TP and HSV1/2 infection. Sensitivity and specificity in secretion samples for TP were 91.7% and 100%; for HSV1, 100% and 98%; and for HSV2 89.7% and 100%. This method seems to be effective in patients suspected of primary TP infection but negative for non-treponemal antibody testing, and this method is useful for the differential diagnosis of new genital, perianal, and oral skin lesions in patients with a history of previous syphilis (12). Along with the present research, in the study of Fabiana et al, in 2020 despite the increase in the use of molecular diagnostic methods to diagnose sexually transmitted infections, cytological findings in Pap smears of patients with pathogens that can only be identified by PCR were evaluated. Cervical samples for conventional and liquid cytology and multiplex PCR were collected from women aged 23 to 54 who underwent

routine screening in the gynecology department. Multiplex PCR was positive in 36.2% of samples. *Ureaplasma parvum* 14.9%, *Chlamydia trachomatis* 10.6%, *Trichomonas vaginalis* 10.6%, *Mycoplasma hominis* 8.5%, *Ureaplasma urealyticum* 4.2%, *Mycoplasma genitalium* 4.2% and 2.2 % *Neisseria gonorrhea*. Multiple pathogens were observed in 12.8% of the samples (13).

In line with our study, Rosas and his colleagues conducted a study in 2021 to investigate the prevalence of *Candida spp, Ureaplasma spp, Trichomonas vaginalis, Neisseria gonorrhoeae, Chlamydia trachomatis, HSV and Mycoplasma*. Their prospective, cross-sectional study included 377 women participating in the reproductive health campaign. Cervicovaginal samples were collected and analyzed with an in-house multiplex PCR to identify *Candida spp, Ureaplasma, Trichomonas vaginalis, Neisseria gonorrhoeae, HSV, Mycoplasma sppand Chlamydia trachomatis*. The most common pathogen identified in this population was *Ureaplasma spp*. 29.4%, followed by *Mycoplasma spp* 14.9%, Candida spp, 12.5%. Also, 33.7% of positive cases were single infections and 12.7% were simultaneous infections. The multiplex PCR method was designed by targeting nucleotide sequences (14). In 2020, Neena and her colleagues conducted a study to investigate chlamydia infection during pregnancy by PCR method. Endocervical swabs were collected from 300 pregnant women. Among them, 29 samples were positive based on PCR. The results showed that the prevalence of *chlamydia trachomatis* in their population was 10%. Hence, it should be considered an important public health problem, especially among sexually active young women of reproductive age. Timely diagnosis and quick treatment of chlamydia infection during pregnancy can eliminate its adverse consequences (15). According to the results of our study, the multiplex method is a fast and cost-effective approach for diagnosis in a clinical laboratory. In this study, co-infections were detected in the least amount of time and cost, which is more cost-effective than single PCR and the detection speed of co-infections is faster.

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Author contributions

Conceptualization: M. R.Z

Methodology: M.B., S. S. A

Writing—original draft preparation: P.SH

Statistical Analysis: A. M

Supervision: M. R.Z

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Availability of data and materials

Data from the present study are available upon reasonable request from the corresponding author.

Ethics approval

The study protocol was approved by the Ethics Committee of the Islamic Azad University, Qom Branch, Isfahan, Iran (Ethical code: R.IAU.QOM.REC.1403.101).

Consent to Participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflict of interest to disclose.

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