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Research Paper

Design a Multiplex PCR Molecular Technique to Detect Sexually Transmitted Agents, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, Herpes Simplex Virus Type 2, and Human Papillomavirus

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ABSTRACT

Introduction: 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 lead to problems such as infertility, ectopic pregnancy, and the risk of genital cancers. Therefore, quick diagnosis of sexually transmitted agents is important. In recent decades, the detection of microbial agents has been improved by using molecular techniques, because it is challenging and often impossible to isolate a disease agent from clinical samples simultaneously and quickly. Most unsuccessful cases and time-consuming culture-based methods have led to the non-identification of microbial agents. This study aims to design a multiplex PCR technique for detecting sexually transmitted agents, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, herpes simplex virus type 2 (HSV-2), and human papillomavirus (HPV) in 2022 in Qom, Iran.

Materials & Methods: In the current study, about 100 Pap smear samples of patients in Qom City, Iran, were evaluated over a one-year period (2022) for testing HSV-2, HPV, *N. gonorrhoeae*, *C. trachomatis*, and *T. vaginalis* using multiplex polymerase chain reaction (PCR) design.

Results: In the investigated samples, the frequency of *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, HSV-2, and HPV was 8%, 5%, 3%, 12%, and 18%, respectively. HPV and *C. trachomatis* agents were found in five samples, and HPV and *T. vaginalis* co-infection was observed in two samples.

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Conclusion: The multiplex PCR 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 Pup smears, effectively speeding up treatment and reducing infection transmission.

1. Introduction

The human urinary tract is a suitable place for the growth of microorganisms such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, Human papillomavirus (HPV), *Mycoplasma genitalium*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, *Haemophilus ducreyi*, *Mycoplasma hominis*, *Treponema pallidum*, *Ureaplasma parvum*, *Candida albicans*, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), HIV, hepatitis B virus (HBV), and *Trichomonas vaginalis* parasite. These microorganisms include bacteria, fungi, viruses, and parasites, which are called sexually transmitted infections (STIs). Globally, STIs are common among youth and adults. Organisms involved in STDs can cause infections in the human genitourinary tract, leading to infertility, pelvic inflammatory disease (PID), miscarriage, and inflammation of the cervix in women and epididymitis, urethritis, and prostatitis in men [1-3].

According to reports, *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis*, *M. hominis*, *U. urealyticum*, *U. parvum*, 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 reduce STD-related genital damage and improve women's health worldwide [4-6].

Annual reports of the World Health Organization (WHO) indicate that approximately 340 million STIs 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, STIs 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 polymerase chain reaction (PCR) molecular technique for the detection of sexually transmitted agents such as *N. gonorrhoeae*, *C. trachomatis*, *T. 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, sampling was done from patients who were referred to Qom, Iran for the presence of HSV-2, HPV, *N. gonorrhoeae*, *C. trachomatis*, and *T. vaginalis*. According to Cochran's formula (Equation 1) to calculate the sample size, about 79 samples with a confidence level of 1.96 should be tested, and 100 samples were tested in this study with a 10% probability of error.

$$1.\ n=\frac{z^2_{1-\alpha/2}\,(P)\,(1-P)}{d^2}$$

2.2. Design of a multiplex PCR molecular technique to detect *N. gonorrhoeae, C. trachomatis, T. vaginalis*, HSV-2, and HPV

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

Primers were designed using CLC Sequence Viewer version 6 [9] and Gene Runner software [10] and the NCBI website [11] (Table 1). First, the target sequence for each of these genes was downloaded from the NCBI database using the CLC Sequence Viewer version 6 software. These sequences were placed one below the other and primers were designed based on the completely conserved regions. Finally, with the help of Gene Runner software version 6.5.52 beta [10], the thermodynamic properties of the primers were checked to ensure that secondary structure (primer dimer, loops, and hairpin structures) were not formed. For the multiplex reaction to be carried out, an effort was made to align the reaction temperature for each of these agents so that detection could be performed in a single run.

Table 1. The sequences of the primers used

Microorganism	Target Gene	Primer Name	Primer Sequence (5´-3´)	Tm (°C)	Product (bp)
C. trachomatis	Phospholipase D endonuclease superfamily	F	TTTTAAACCTCCGGAACCC	51	347 bp
		R	GCATCGCATAGCATTCTTTG	51.8	
N. gonorrhoeae	*porA* pseudo gene	F	GTTGCGAATCCGTTTGGC	52	592 bp
		R	CGAAACCATGGGCATAGC	51.8	
T. vaginalis	Adhesive protein gene	F	CATGCCTTGTCCAGTTCGA	51	248 bp
		R	GCGGGAAACAGCCATATC	51	
HSV2	Glycoprotein D (*US6*) gene	F	CCTGCTAGTTGTCGCGGT	51	697 bp
		R	ATGCTGTCGACCGTCACG	51	
HPV	*E6*	F	CGTCCM*ARR*GGAW*ACTGATC	51	450 bp
		R	GCMCAGGGWCATAAY*AATGG	51	

*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, a final volume of 25 µL contained a mixture of 12.5 µL Mastermix (SinaClon Co., Iran), 1 µL of each primer, 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 cycles of denaturation at 95 ˚C for 30 sec, annealing at 51 ˚C for 30 sec, extension at 72 ˚C for 40 sec, and final extension at 72 ˚C for 5 min. Finally, 5 µL of each PCR product was loaded into the wells of a 2% electrophoresis gel and placed in the electrophoresis tank for 50 min, and the results were observed with the gel doc.

3. Results

During this research, about 100 samples of patients referred for HSV, HPV, *N. gonorrhoeae*, *C. trachomatis*, and *T. vaginalis* tests were prepared for medical diagnosis laboratories after receiving consent. After extracting the sample, qualitative and quantitative control of extraction was performed. Then, based on the primers designed for each PCR target, and after the set-up, a multiplex PCR test was performed. Based on this, in the multiplex PCR technique, bands were observed as follows: *T. vaginalis* at 248 bp, *C. trachomatis* at 347 bp, HPV at 450 bp, *N. gonorrhoeae* at 592 bp, and HSV-2 at 697 bp (Figure 1).

3.1. Frequency of pathogenic agents in the samples

In this study, the frequency of *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, HSV-2, 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 GA STD12 Plus RT-PCR KIT (GeneovA co., Iran), and the results were 100% consistent.

HPV and *C. trachomatis* were detected in five samples, and HPV and *T. vaginalis* were detected in two samples.

4. Discussion

In this research, about 100 Pap smear samples were used to detect HSV, HPV, *N. gonorrhoeae*, *C. trachomatis*, and *T. vaginalis* using the designed multiplex PCR technique. In this study, the frequency of *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, HSV-2, and HPV was 8%, 5%, 3%, 12%, and 18%, respectively. The samples were checked simultaneously with the molecular diagnosis kit of GeneovA Company, and the positive cases were confirmed with the kit based on real-time PCR. Also, the results showed that HPV and *C. trachomatis* were detected together in five samples, and HPV and *T. vaginalis* infections were observed in two samples.

In the study by Faroughi and Amini (2021), which was conducted on 60 infertile patients with symptomatic vaginal infection who were referred to Kerman Hospital, the frequency of infection with *N. gonorrhoeae* and *Toxoplasma gondii* was 6.6% and 10%, respectively. Co-infection with *N. gonorrhoeae* and *T. gondii* was not detected in any of the samples [12]. The results of Amini et al study showed that the multiplex PCR method

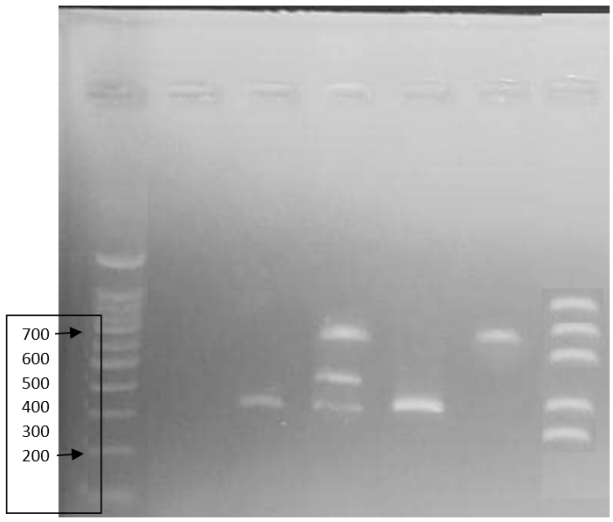

Figure 1. Multiplex PCR for samples of *T. vaginalis* (248 bp), *C. trachomatis* (347 bp), HPV (450 bp), *N. gonorrhoeae* (592 bp), and HSV-2 (697 bp)

was suitable for the diagnosis of *N. gonorrhoeae* and *T. gondii* in vaginal infections, which is consistent with the results obtained in the present study [12]. In the study of Kriesel et al. (2016), an STI panel including several sets of PCR primers for each organism was designed for the detection of *C. trachomatis*, *N. gonorrhoeae*, *T. pallidum*, *T. vaginalis*, *M. genitalium*, *U. urealyticum*, *H. ducreyi*, and different types of herpes viruses. Among the samples, 13% *C. trachomatis*, 7% *N. gonorrhoeae*, 3% *T. vaginalis*, 2% HSV-2, 12% *U. urealyticum*, 3% *M. genitalium*, and 4% *T. pallidum* were detected. The concordance between the FilmArray STI panel and the standard nucleic acid amplification test was 98% for *C. trachomatis* and 97% for *N. gonorrhoeae* [13].

Beayni et al. (2021), 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 the samples were positive for one or more organisms. *U. urealyticum/parvum* was the most common pathogen (49.3%), followed by *G. vaginalis* (33.5%), *C. trachomatis* (5.36%), *M. genitalium* (5.16%), *N. gonorrhoeae* (2.5%), HSV (2.5%), and *T. vaginalis* (1.39%). In terms of pathogen distribution between genders, *U. urealyticum/parvum*, HSV, and *G. vaginalis* were more common in women, and the rest were more common in men [14].

So, with the development of molecular techniques, STD screening with high sensitivity and specificity became easier. Yuan et al. (2023) created a TP-HSV1-HSV2 multiplex polymerase chain reaction by targeting

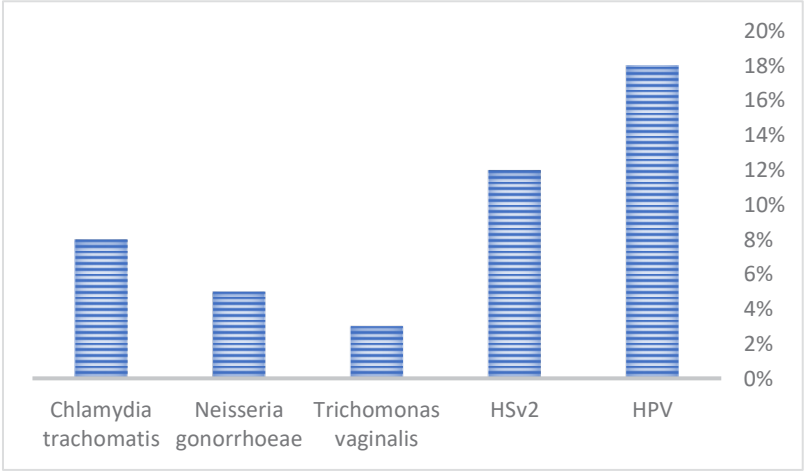

Figure 2. Frequency of pathogenic agents

the conserved regions of the *PolA* gene and the *UL42* gene of HSV-1 and HSV-2 to detect the skin lesions of 115 patients suspected of TP and HSV-1/2 infection. Sensitivity and specificity in secretion samples for TP were 91.7% and 100%; for *i*, 100% and 98%; and for HSV-2 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 it is useful for the differential diagnosis of new genital, perianal, and oral skin lesions in patients with a history of previous syphilis [15]. Along with the present research, in the study of Carneiro et al. (2020), despite the increase in the use of molecular diagnostic methods to diagnose STIs, 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. *U. parvum* (14.9%), *C. trachomatis* (10.6%), *T. vaginalis* (10.6%), *M. hominis* (8.5%), *U. urealyticum* (4.2%), *M. genitalium* (4.2%), and *N. gonorrhoeae* (2.2%) were detected. Multiple pathogens were observed in 12.8% of the samples [16].

In line with our study, Hernández-Rosas and his colleagues conducted a study in 2021 to investigate the prevalence of *Candida* spp., *Ureaplasma* spp., *T. vaginalis*, *N. gonorrhoeae*, *C. 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*, *T. vaginalis*, *N. gonorrhoeae*, HSV, *Mycoplasma* spp., and *C. trachomatis*. The most common pathogen identified in this population was *Ureaplasma* spp. (29.4%), followed by *Mycoplasma* spp. (14.9%) and *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 [17]. In 2020, Neena and her colleagues conducted a study to investigate *C. trachomatis* infection during pregnancy by the 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 *C. 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 *C. trachomatis* infection during pregnancy can eliminate its adverse consequences [18].

5. Conclusion

According to the results of this 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 at lowest cost, which is more cost-effective than single PCR, and the detection speed of co-infections was faster.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was approved by the Research Ethics Committee of Qom Branch, Islamic Azad University, Qom, Iran (Code: IR.IAU.QOM.REC.1403.101).

Data availability

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

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Authors' contributions

Conceptualization and supervision: Mohammad Reza Zolfaghari; Statistical analysis: Abbas Morovvati; Methodology and experiments: Marziyeh Bastamifard and Seyed Soheil Aghaei; Writing: Pegah Shakib.

Conflict of interest

The authors declared no conflict of interest.

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References

[1] Sadeqi S, Nikkhahi F, Javadi A, Eskandarion S, Amin Marashi SM. Development of multiplex real-time quantitative PCR for simultaneous detection of Chlamydia trachomatis, Mycoplasma hominis, Ureaplasma urealyticum, and Mycoplasma genitalium in infertile women. Indian J Med Microbiol. 2022; 40(2):231-4. [DOI:10.1016/j.ijmmb.2022.01.011] [PMID]

[2] de Souza LS, Sardinha JC, Talhari S, Heibel M, Santos MND, Talhari C. Main etiological agents identified in 170 men with urethritis attended at the Fundação Alfredo da Matta, Manaus, Amazonas, Brazil. An Bras Dermatol. 2021; 96(2):176-83. [DOI:10.1016/j.abd.2020.07.007] [PMID]

[3] Kılıç M, Beşli Y, Köseoğlu E, Palaoğlu EK, Esen T. Gardnerella vaginalis: Is it an Underestimated Cause of Urinary Symptoms in Males? Infect Dis Clin Microbiol. 2022; 4(3):172-7.[DOI:10.36519/idcm.2022.172] [PMID]

[4] Garcia MR, Leslie SW, Wray AA. Sexually Transmitted Infections. 2024 Apr 20. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan. [PMID]

[5] de Souza LS, Sardinha JC, Talhari S, Heibel M, Santos MND, Talhari C. Main etiological agents identified in 170 men with urethritis attended at the Fundação Alfredo da Matta, Manaus, Amazonas, Brazil. An Bras Dermatol. 2021; 96(2):176-83. [DOI:10.1016/j.abd.2020.07.007] [PMID]

[6] Carneiro FP, Darós AC, Darós ACM, de Castro TMML, de Vasconcelos Carneiro M, Fidelis CR, et al. Cervical Cytology of Samples with Ureaplasma urealyticum, Ureaplasma parvum, Chlamydia trachomatis, Trichomonas vaginalis, Mycoplasma hominis, and Neisseria gonorrhoeae Detected by Multiplex PCR. Biomed Res Int. 2020; 2020:7045217. [DOI:10.1155/2020/7045217] [PMID]

[7] Suehiro TT, Gimenes F, Souza RP, Taura SKI, Cestari RCC, Irie MMT, et al. High molecular prevalence of HPV and other sexually transmitted infections in a population of asymptomatic women who work or study at a Brazilian university. Rev Inst Med Trop Sao Paulo. 2021; 63:e1. [DOI:10.1590/s1678-9946202163001] [PMID]

[8] Ma W, Chen Z, Niu S. Advances and challenges in sexually transmitted infections prevention among men who have sex with men in Asia. Curr Opin Infect Dis. 2023; 36(1):26-34. [DOI:10.1097/QCO.0000000000000892] [PMID]

[9] No Author. CLC Sequence Viewer version 6 [Internet]. 2026 [26 January 2026]. Available from: [Link]

[10] No Author. Gene Runner software version 6.5.52 beta [Internet]. 2026 [26 January 2026]. Available from: [Link]

[11] No Author. NCBI website [Internet]. 2026 [26 January 2026]. Available from: [Link]

[12] Faroughi E, Amini K. [Molecular identification of Neisseria gonorrhoeae and Toxoplasma gondii isolated from infertile women with vaginal swab samples by Multiplex-PCR (Persian)]. Alborz Uni Med J. 2021; 10(3):297-304. [Link[

[13] Kriesel JD, Bhatia AS, Barrus C, Vaughn M, Gardner J, Crisp RJ. Multiplex PCR testing for nine different sexually transmitted infections. Int J STD AIDS. 2016; 27(14):1275-82. [DOI:10.1177/0956462415615775] [PMID]

[14] Beayni NE, Hamad L, Nakad C, Keleshian S, Yazbek SN, Mahfouz R. Molecular prevalence of eight different sexually transmitted infections in a Lebanese major tertiary care center: impact on public health. Int J Mol Epidemiol Genet. 2021; 12(2):16-23. [PMID]

[15] Yuan L, Xia D, Zhou Q, Xu W, Xu S, Yin Y. An evaluation of a multiplex PCR assay for the detection of Treponema pallidum, HSV-1, and HSV-2. Diagnos Microbiol Infect Dis.2023; 106(3):115958. [DOI:10.1016/j.diagmicrobio.2023.115958] [PMID]

[16] Carneiro FP, Darós AC, Darós ACM, de Castro TMML, de Vasconcelos Carneiro M, Fidelis CR, et al. Cervical cytology of samples with ureaplasma urealyticum, ureaplasma parvum, chlamydia trachomatis, trichomonas vaginalis, mycoplasma hominis, and neisseria gonorrhoeae detected by multiplex PCR. Biomed Res Int. 2020; 2020:7045217. [DOI:10.1155/2020/7045217] [PMID]

[17] Hernández-Rosas F, Rey-Barrera M, Conejo-Saucedo U, Orozco-Hernández E, Maza-Sánchez L, Navarro-Vidal E, et al. Monitoring sexually transmitted infections in cervicovaginal exfoliative samples in Mexican Women. Pathogens. 2021; 10(12):1618. [DOI:10.3390/pathogens10121618] [PMID]

[18] Neena A, Deepa R. Detection of chlamydia trachomatis infection among the pregnant women attending a tertiary care hospital in Kerala - South India by polymerase chain reaction. Indian J Med Microbiol. 2020; 38(3 & 4):319-23. [DOI:10.4103/ijmm.IJMM_19_429] [PMID]