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RESEARCH ARTICLE

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Development a rapid and accurate multiplex real time PCR method for the detection *Chlamydia trachomatis* and *Mycoplasma hominis*

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Background: Sexually transmitted diseases easily spread among sexually active people and often have no symptoms. Rapid and accurate method for detecting these infections are necessary in early stages. The traditional detection methods of them are difficult and time—consuming.
Methods: In this study, multiplex real time PCR was optimized for rapid identification of *Chlamydia trachomatis* and *Mycoplasma hominis* in a single tube and was performed with our designed primers. The sensitivity test was carried out to designed primers with diluted genomic DNA. To defined the specificity, non STD bacteria were used as DNA template.
Results: This study indicated that the developed multiplex real time PCR can be an effective alternative procedure to the conventional methods for rapid and accurate identification of C *Chlamydia trachomatis* and *Mycoplasma hominis*. Multiplex real-time PCR Results of them were checked with melting curves. The sensitivity of our designed primer by multiplex real time PCR for *Chlamydia trachomatis* and *Mycoplasma hominis* were 4.78×10^{10} and 8.35×10^{10}, respectively, Which the primers did not amplify any product from a non-STD species.
Conclusions: Multiplex real time PCR by our new primers and analysis of melting curves were successfully usable for rapid and accurate detection of *Chlamydia trachomatis* and *Mycoplasma hominis*. This assay instead of traditional culture method, has considerable potential to be rapid, accurate and highly sensitive molecular diagnostic tool for simultaneous and direct detection.

KEYWORDS
Chlamydia trachomatis, multiplex real time PCR, *Mycoplasma hominis*

1 | INTRODUCTION

Sexually transmitted diseases are common infections among sexually active youth and adolescents.[1] Risky behavior of individuals lead to an increased risk of transmitted infections. Nowadays, despite development of diagnostic tests and treatment, they are still the major concern of clinicians around the world.[2,3] *Chlamydia trachomatis* and *Mycoplasma hominis* are the most common infections than other STDs.[4,5] The most cases of *C. trachomatis* and *M. hominis* infections are asymptomatic and cannot be detected accurately.[6] Approximately 70%-80% of *C. trachomatis* infections are undetectable, because they often have no symptoms.[2,4] Also, *M. hominis* can be separated from genital tract infections of 35% of asymptomatic men and women.[7,8]

Chlamydia trachomatis is a gram-negative bacterium, non-motile, pleomorphic, an obligate intracellular human pathogen, and 18 different serotypes of them are known. Serotype D through to K are associated with genital tract infections.[5,9] *Mycoplasma hominis* is an intracellular gram-negative bacterium, pleomorphic, 0.2-0.3 μm in

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diameter, and is one of the smallest bacteria without cell wall, which instead of it, contains a three-layered membrane including sterol. Also, this bacterium is capable of parasitic and saprophytic existence, and able to self-replication.[8-10]

These two bacteria are able to cause genital tract infections and non gonococcal urethritis.[8-12] These infections may develop urethritis, proctitis and pharyngitis in both males and females and on the other hand, in female may cause ectopic pregnancy, abortion, and pelvic inflammation disease. Moreover, they have the potential for transferring to infant.[4,6,7,13] There is a widespread belief that *Chlamydia* infections increase the risk of cervical cancer.[14] The role of *M. hominis* is in pelvic inflammatory disease, bacterial vaginosis, and can be attached to the head, middle, and tail of sperm (required to obtain steroids), and causes sperm immobility and even penetrates into the sperm.[15-18] Bacterial detection of *C. trachomatis* is difficult using conventional method.[3,19,20] Also, the conventional identification of *M. hominis* is culture on complex medium which is time-consuming.[4] Nowadays, improvements in the molecular detection technologies have produced more deeply and sensitively than conventional methods in the laboratory.[21-23] Recently, multiplex real time PCR method is used due to high sensitivity and specificity as a routine test for diagnosis of infectious in the clinical microbiology laboratories.[24,25] In this survey, multiplex real-time PCR was optimized for rapid identification of *C. trachomatis* and *M. hominis* in single tube.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens

A total of 200 endocervix samples were aseptically collected using Dacron swabs by the gynecologists from symptomatic women who referred to the Women Reference Hospital, (Tehran, Iran) during a period of 12 months from June 2013 to June 2014. Swabs were inoculated into tubes containing PPLO(pleuropneumonia-like organisms) broth as transport medium for *M. hominis* and 2-SP(2-sucrose phosphate) transport medium for *C. trachomatis*. Then, swabs were placed in phosphate buffer solution(PBS) and immediately frozen at −70°C for later PCR assays.

2.2 | *Mycoplasma homonis* culture

The samples were filtered through 0.45 μm filter membrane and were inoculated in specific arginine PPLO broth medium (Biolife, Italy) then, were incubated at 37°C for 5 days. Inoculated medium broth was checked out for color changes daily, from yellow to purple. Next, suspected samples were cultured on arginine PPLO agar and were incubated at 37°C for 5-7 days, until *M. hominis* colonies appear like fried-egg shaped, which directly were observed by microscope.[26-28]

2.3 | Genomic DNA extraction

The swab samples put and frozen in PBS were dissolved, and DNA extractions were done according to the manufacturer's instructions (Bioflux, China). To determine concentrations and purity of extracted DNA, UV- photometer (Nanolytik, Germany) was used at 260 and 280 nm. Purified DNA was stocked at −70°C until further analysis.

2.4 | Primer designing and in silico confirmation

DNA targets for primer designing included: *gap* gene of *M. hominis* and *momp* gene of *C. trachomatis* which were deposited in NCBI database. Conserved regions were selected for primer designing, after aligning of 35 of target sequences by bioedit multiple alignment software (Bioedit 7.1, An Abbott company, Carlsbad, CA, USA). Also, oligonucleotide primers were designed using gene runner software (version 4, Helio Genetics Corporation, East Hanover, NJ, USA). Then, designed primers were confirmed by primer and nucleotide BLAST tools (National Center for Biotechnology Information). The primer sequences, their amplicon lengths, and melting temperature (Tm) values are provided in Table 1.

2.5 | Run PCR for detection two bacteria

The PCR reaction was set up using positive control DNA from *C. trachomatis* ATCC VR628T and *M. hominis* ATCC 23114 for the blank. *C. trachomatis* and *M. hominis* were detected by PCR reaction. The conventional single PCR assay was carried out for these two pathogens. Then, the duplex PCR reactions were also done and the PCR amplification conditions were same as that of the assay accordance with the manufacturer's protocol. Amplification reactions were accomplished in a final volume of 25 μL, containing 5 μL(5-10 ng/μL) of template DNA, 12.5 μL of the Master mix PCR solution (Ampliqon, Odense, Denmark), and 1 μL (10 pmol/μL) of each reverse and forward primer. The reaction mixture was incubated for 5 minutes at 94°C, then followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C, and a final elongation step of 5 minutes at 72°C. The PCR products were run on 2.0% agarose gel electrophoresis with DNA size marker (100 bp) (Thermo Fisher Scientific, Waltham, MA,

TABLE 1 Primers used for detection *Chlamydia trachomatis*, and *Mycoplasma hominis* by Real Time PCR

Species	Target gene	Sequence	Product size	Amplicon Tm
C. trachomatis	*momp*	F:TGGAGTTAAATGGTCTCGAGC R:GATTTCATCTTGTTCAATTGCA	194 bp	85.5°C
M. hominis	*gap*	F:TGGTTCCAACAACAACAGG R:CGAATGATCCAGTTATTGTTG	111 bp	81.5°C

USA) to confirm the size of amplicons. Finally, real time PCR products were purified and sequenced (Macrogen, Seoul, South Korea) for confirming of amplified regions.

2.6 | Detection of target bacteria by real-time PCR

Real time PCR was performed using SYBR green PCR (Ampliqon) and Exicycler 96 (Bioneer, Daejeon, South Korea). Reaction mixture contained: 1.0 μL of each primer (10 pmol/μL), 12.5 μL SYBR green I PCR master mix, 2 μL of purified DNA (5 ng/μL),and 8.5 μL free water. The real time PCR was done in 25 μL final volume. The reaction protocol was accomplished as following: an initial step of 5 minutes at 95°C, a 40 amplification cycle consisting of 95°C for 15 seconds, and 60°C for 30 seconds. We used three targets that were amplified in a single reaction: the major outer membrane protein (*momp*) gene for detection of *C. trachomatis*, glyceraldehyde-3-phosphate dehydrogenase (*gap*) housekeeping gene for identification of *M. hominis*, and an internal control [29] (16S rRNA). At the end of the amplification, melting temperature program was performed by ramping from 65°C to 90°C at 0.1°C/s and fluorescence was measured during each cycle. Next, the melting curves of the real time PCR products of *C. trachomatis* and *M. hominis* were analyzed. Individual real time PCR assays were done prior to multiplexing for all the three primer pairs to record their exact melt peaks.

Two hundred clinical samples were obtained from symptomatic women who referred to the Women Reference Hospital, (Tehran, Iran). Detection of bacteria were carried out by standard tests which include: Gram stain which followed by specific biochemical tests for *M. hominis*. Afterward, the swab samples put and frozen in PBS were dissolved, and DNA extractions were accomplished, according to the manufacturer's instructions (Bioflux, Bidong Island, China). Then Multiplex real time PCR was performed.

2.7 | Determination of sensitivity and specificity of real time PCR

To confirm the sensitivity and specificity of the test, the positive and negative control specimens were also blindly re-examined. Evaluation of the sensitivity of test for current designed primer was performed by real time PCR with diluted genomic DNA in 100 μg, 10 μg, 100 ng, 10 ng, and 100 pg concentrations. For specificity, bacterial DNA of *Lactobacillus acidophilus*, *Gardnerella vaginalis*, and *Streptococcus agalactiae* were used as template in real time PCR.

3 | RESULTS

3.1 | Conventional PCR assay

Conventional PCR was performed as a duplex to show the different gene amplicon sizes of the two organisms. The size of *C. trachomatis momp* gene amplicon and *M. hominis gap* gene amplicon are 194 bp and 111 bp, respectively. Also, Conventional multiplex PCR was carried out for two organisms. PCR amplicons were confirmed by sequencing and aligning with NCBI database using BLAST.

3.2 | Multiplex real time PCR assay

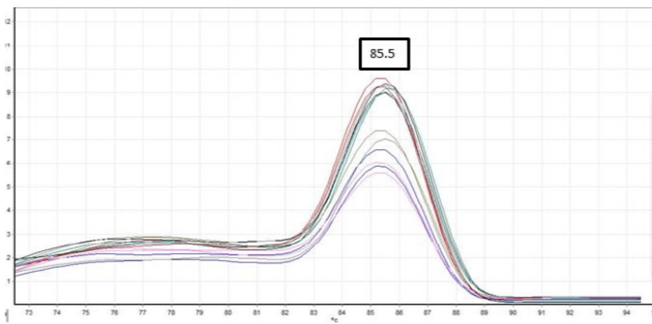
Rapid detection of *C. trachomatis* and *M. hominis* was developed by multiplex real time PCR method and were identified based on the melt curve analysis. New designed primers to the detection of these bacteria were individually examined with crude DNA of *C. trachomatis* and *M. hominis*. The developed multiplex real time PCR in this survey can be used as a simple and useful alternative to the conventional methods for rapid and accurate identification of *C. trachomatis* and *M. hominis* (Figure 1C). Also, SYBR Green Real time PCR was first performed for each of the two genes with their respective primers in separate reaction tubes. The multiplex reaction was optimized in two studied genes and were identified based on the melting curve analysis and represented two peaks: In reaction 1 *C. trachomatis* (Figure 1A) represented a major peak with Tm value 85.5. In reaction 2, *M. hominis* represented a small peak with Tm value 81.5 (Figure 1B). The PCR products were analyzed by agarose gel electrophoresis.

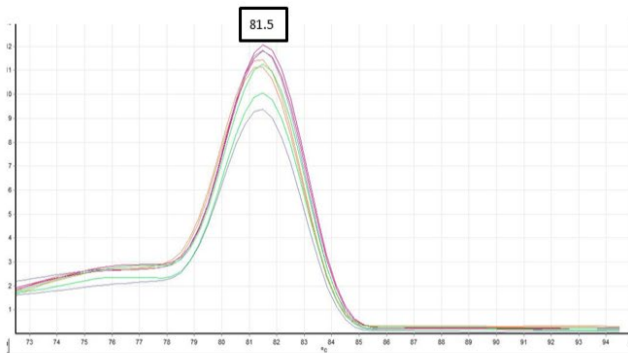
3.3 | Detection of STD pathogens in clinical samples

Multiplex real time PCR was performed on 200 clinical specimens. Forty of them showed amplification curves and melt peaks for *C. trachomatis* and *M. hominis*. The end, the sensitivity of our designed primer by multiplex real time PCR for *C. trachomatis* and *M. hominis* were 4.78×10^{10} and 8.35×10^{10}, respectively which the primers did not amplify any product from a non-STD species.

4 | DISCUSSION

Since STDs are on the rise and symptoms may not appear immediately, infections and complications of them are not limited to patients thus, can be transmitted to other people. Furthermore, in pregnancy can be passed to infant, which the possible consequences can occur such as spontaneous abortion, premature birth, stillbirth, intrauterine death, low birth weight, eye and lung infections in babies.[4,27,30] Also, in the cases of lack of treatment, there might be serious complications such as infertility, ectopic pregnancy, cervical cancer, uterine rupture occur bleeding, and ultimately causing death. According to the World Health Organization report, approximately 90 million people infect annually with *C. trachomatis* around the world and this infection is more common among other cases of STDs.[6,31] Also, *M. hominis* is the most common genital mycoplasma which causes genitourinary syndromes and may induce perinatal infections.[13] Therefore, detection of these bacteria from infected women has special significance. According to the mentioned effects of genital diseases, prevention, screening, and treatment are extremely important. The *C. trachomatis* is able to grow in cell culture, which is time-consuming and costly.[19,20,32] Moreover, *M. hominis* culture which is considered as gold standard method in clinical laboratory, is time-consuming, requires live organism, and may yield ambiguous results.[4] Molecular methods can be a useful tool to detect these two bacteria. The main advantages of molecular techniques for detecting of STDs are high sensitivity and specificity, lower response time, simultaneous





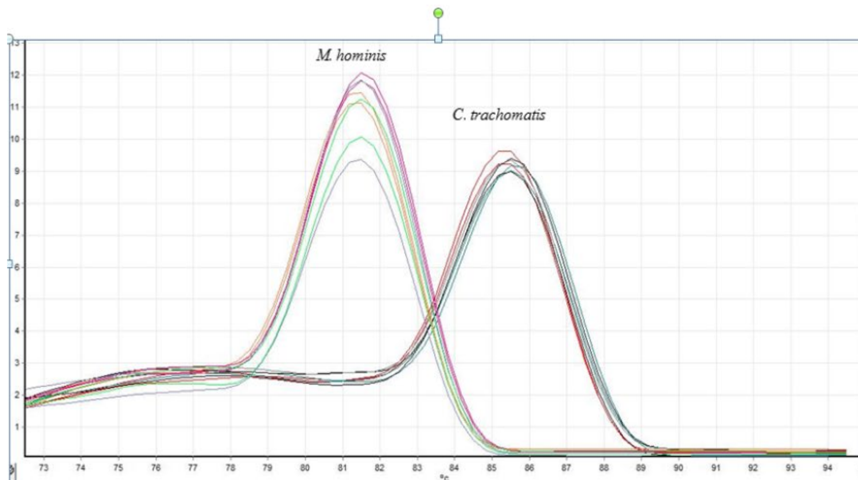


FIGURE 1 Melting curve analysis of multiplex real-time PCR. Melting curves for the selected genes of the 2 STDs in a singleplex PCR reaction for *Chlamydia trachomatis* (A), *Mycoplasma hominis* (B) and Duplex of *C. trachomatis* and *M. hominis* (C)

detection of several pathogens, lower cost for routine use, easy samples collection, and screening.[12, 21-23, 33] According to the role of *C. trachomatis* and *M. hominis* in genital tract infections, their detection is extremely important. The results of this study illustrated that the designed primers have a high degree of sensitivity and specificity for detection and identification of these two bacteria in genital tract infections.

The multiplex real time PCR method was used for early detection of specific target species to prevent transmission and treatment of disease, due to time-consuming process of gold standard procedures and difficult diagnostic of some fastidious species. The real time PCR has several advantages such as: rapid availability of results, high sensitivity and specificity also, no post-PCR contamination in compare with traditional PCR. Different based on melting curve is a powerful tool to distinguish different sequences.[34] Furthermore, real time PCR which is used in clinical diagnostics and the multiplex assay, can be valuable for the detection of *C. trachomatis* and *M. hominis* infections.

Recently, several researchers used in-house or commercial PCR assays to identify STD bacteria by detecting the *16S rDNA* gene or other genes in s-PCR or m-PCR.[35,36] The purpose of our study was to develop multiplex real time PCR to rapid and accurate detection of STDs, with selection of appropriate target genes and oligonucleotide primers, in the simultaneous amplification of these target genes in two reaction tubes, under same PCR condition. We used the major outer membrane protein (*momp*) gene for detection of *C. trachomatis* and glyceraldehyde-3-phosphate dehydrogenase (*gap*) housekeeping gene for identification of *M. hominis*.

In a study by Aguilera-Arreola et al.[35] in mexico city by multiplex PCR and designed primer that amplify species specific 16S rDNA target regions of *C. trachomatis* (402 bp) *Neisseria gonorrhoeae* (694 bp), *M. hominis* (604 bp) and *Ureaplasma urealyticum* (898 bp) from genomic DNA.While we used multiplex real time PCR method by designed primers to *gap* gene for *M. hominis* (111 bp) and *momp* gene for *C. trachomatis*) 194 bp). They have reported that for the evaluation of the specificity of the multiplex PCR assay, the strains of *S. agalactiae Staphylococcus saprophyticus*, *L. acidophilus*, *G. vaginalis*, *and N. gonorrhoeae* were applied as DNA template, which the primers did not amplify any product from a non-STD species, that this result was almost similar to our finding. Also, the result of determination of sensitivity by serial dilution DNA for *C. trachomatis* and *M. hominis* were 5.12×10^5 and 61.19×10^6 copies of a DNA template, respectively.[35] Whereas, the sensitivity of our designed primer by multiplex real time PCR for *C. trachomatis* and *M. hominis* were 4.78×10^{10} and 8.35×10^{10}, respectively.

This difference results occurred because of the used target DNA as a template for amplification, sequence primer, lengh amplicication, and used method to amplify DNA.

In a study by Rodrigues et al.[36] s-PCR was performed by target sequence cryptic plasmid, *cppB*, major adhesion protein gene, urease gene for *C. trachomatis*, *N. gonorrhoeae*, *Mycoplasma genitalium*, *U. urealyticum*, respectively, in five different reactions. whereas we used *gap* gene for *M. hominis* and *momp* gene for *C. trachomatis* in designing primers in our study and demonstrated that the muitiplex realtime PCR technique can be used for the simultaneous detection of two bacteria. We have compared the real time PCR results with the conventional culture techniques and have shown it, as a reliable method of accurately identifying the pathogens which it does not require the unique expertise, while the conventional culture methods require to a technical expertise. In conclusion, real time PCR was successfully developed by designing specific primer and analysis of melting curves. It is illustrated that a multiplex real time PCR based on different genes using two primer sets that provide reliable means for detection of *C. trachomatis* and *M. hominis*. Also, it has the potential to be used in microbiology laboratories and can be used as an alternative method for the routine microbiological analysis and the treatment.

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