

Evaluation of Loop-Mediated Isothermal Amplification Assay for Detection of *Mycoplasma hominis* in Vaginal Samples in Comparison with Real Time PCR

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Abstract

Mycoplasma hominis as one of the etiologic agents of non-gonococcal urethritis in women should be identified for early treatment that led to the patient does not suffer from serious complications such as pelvic inflammatory disease and infertility. With regard to the importance of *M. hominis* in genitourinary diseases, is required for the choice of the appropriate, reliable, simple, and repeatable laboratory assay for diagnosis. The focus of this study was to investigate 16srRNA-based LAMP assay for the identification of *M. hominis* from vaginal swabs in comparison with quantitative real-time PCR (qPCR). The eighty vaginal swabs were taken and analyzed by cultures, qPCR, and LAMP assay. Totally, 13 (16.3%), 14 (17.5%), and 25 (31.3%) samples were positive for *M. hominis* by culture, qPCR, and LAMP assay, respectively. The results indicate a sensitivity of 100%, a specificity of 83.3%, a negative predictive value (NPV) of 100%, and a Positive predictive value (PPV) of 56% of the developed LAMP assays. According to the result, the LAMP assay is significantly more specific, and sensitive than both qPCR and culture methods for diagnosis of *M. hominis*.

Keywords: *Mycoplasma hominis*, LAMP, vaginal samples, Real-Time PCR

Резюме

Mycoplasma hominis, като един от етиологичните агенти на негонококов уретрит при жени, трябва да бъде идентифициран и да се започне ранно лечение, което ще доведе до това пациентът да не страда от сериозни усложнения като възпалително заболяване на таза и безплодие. Поради важността на *M. hominis* при пикочно-половите заболявания, е необходимо да се избере подходящ, надежден, прост и повторим лабораторен анализ за диагностициране. Фокусът на това проучване е изследването на 16srRNA базиран LAMP анализ за идентифициране на *M. hominis* от вагинални тампони в сравнение с количествен qPCR анализ. От осемдесет вагинални тампона са анализирани общо 13 (16.3%), 14 (17.5%) и 25 (31.3%) проби положителни за *M. hominis* чрез прилагане съответно на метода на културане, както и на qPCR и LAMP анализ. Резултатите показват чувствителност от 100%, специфичност 83.3%, отрицателна прогнозна стойност (NPV) от 100% и положителна прогнозна стойност (PPV) от 56% от разработените LAMP анализи. Според резултатите, LAMP анализът е значително по-специфичен и чувствителен от qPCR и от методите за културане за диагностика на *M. hominis*.

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Introduction

Mycoplasmas are tiny cell-free organisms that are capable of growing in a common culture medium. Among these bacteria, *Mycoplasma hominis* and *Ureaplasma urealyticum* are mother-to-child transmissions during labor and delivery (Moridi *et al.*, 2020). These bacteria are causative agents for endometritis, chorioamnionitis, low birth weight, and postpartum fever. They are also a main cause of pneumonia and meningitis in very low birth weight infants (Luki *et al.*, 1998). It has been implicated in bacterial vaginosis, abortion, cesarean section infection, PID, pyelonephritis, and urethritis (Hossainpour *et al.*, 2022). *M. hominis* is one of the sexually transmitted bacteria found in the genitourinary tract in 21–54% of women and in 4–13% of men (Leli *et al.*, 2018; Moridi *et al.*, 2020). Acquisition of *M. hominis* through the birth canal may cause meningitis, eyes, and brain abscesses in infants (Hossainpour *et al.*, 2022). Bacterial colonization depends on factors such as socioeconomic status, sexual activity, and age (Leli *et al.*, 2018; Zheng *et al.*, 2020). The principal laboratory diagnostic assay for *M. hominis* is culture isolation, but bacterial cultures have problems including bacterial growth and wasting of 2 to 5 days to determine the result of culture, and complex nutritional requirements. The colonies are immersed in agar and microscopic and require the skills and experience of the laboratory staff. Accordingly, Conventional diagnosis of these bacteria is not routinely performed in laboratories (Moridi *et al.*, 2020). Identification of specific serologic responses against genital *Mycoplasma* is also difficult due to heterogeneity and cross-reactivity. Although polymerase chain reaction (PCR) is an accurate assay for detecting small amounts of DNA, it cannot be widely used in clinical practice because of its complexity and the need to use heat cycles and special equipment. On the other hand, false negative results are common due to the presence of PCR inhibitors that can originate from the sample or may be introduced during sample processing or nucleic acid extraction.

To overcome these problems, a gene amplification strategy named Loop-Mediated Isothermal Amplification (LAMP) has been expanded (Campos *et al.*, 2015). Which was first described by Notomi *et al.* (2015) as a promising alternative to conventional PCR assay. In this assay, using Bst DNA polymerase, a large fragment of DNA is synthesized by auto-cycling and translocating the enzyme onto both strands of DNA in 60 minutes. The opacity of the reaction increases proportion-

ally to the amount of DNA synthesis, resulting in the formation of a white precipitate as an indicator for the identification of amplified nucleic acids. Some of the features of the LAMP assay are high specificity, inexpensive, and more rapid than PCR. Also, since LAMP amplification produces a large amount of product, no further gel electrophoresis is required for the final diagnosis and visual (Azizi *et al.*, 2022). LAMP assay has been used to identify *Mycoplasma pneumoniae* in various studies and it has been shown that this assay has sensitivity and specificity by 78.4% and 97.3% in comparison with serologic methods, respectively. The LAMP assay is suitable for the rapid detection of *M. pneumoniae*, but it is not available for the identification of *M. hominis* (Luki *et al.*, 1998). Concerning the importance of *M. hominis* in genitourinary diseases as well as difficulties of culture and PCR in diagnosis of *M. hominis*, there's need for development of inexpensive, reliable, simple and repeatable laboratory methods in diagnosis. Thus, the focus of this study was to investigate the 16srRNA-based LAMP technique in comparison with qPCR for the identification of *M. hominis* from vaginal swabs of women.

Materials and Methods

Clinical sample

Two Dacron polyester swabs in phosphate-buffered saline (PBS) samples were taken from patients referred to the gynecologist in the Hamadan infertility center, Hamadan, Iran. The first swab was cultured in sterile conditions, immersed in 3 ml of PPLO Broth (Merck, Germany) and the second swab, for subsequent molecular steps, was inoculum in 1 ml PBS buffer and finally immediately frozen at -70°C.

Bacterial isolation and growth condition

PPLO Broth and PPLO Agar (Merck, Germany) were used for the culture of samples and the necessary reagents and supplements were added according to the manufacturer's instructions. Arginine hydrolysis in a liquid medium and discoloration from yellow to purple, the appearance of colonies with the appearance of fried eggs, and lack of bacterial observation in smear stained with hot staining are sufficient to identify *M. hominis*. As a positive control, *M. hominis* PG21 (ATCC23114) was used.

DNA Extraction

DNA extraction from clinical specimens was performed by DNA extraction kit (Cinna Gen Co,

Iran) according to the manufacturer's manual. The purity of the DNA was determined by the Nano drop ND-1000 spectrophotometer (Nano drop Technologies, Inc., Wilmington, DE, USA). The DNA samples were stored at -70°C until further analysis.

Quantitative real-time PCR reaction

qPCR was performed according to the following protocol and using the specific primers which amplify a 101 bp part of the 16S rRNA-encoding gene (Table 1). Use 10 µl of the Taq 2x Master Mix (Applied Biosystem), in a final volume of 20 µl, Add 2 µl of each 20 µM primer, 1 µL probe, and 5 µL DNA template. The qPCR protocol was 95°C for 5 min, 95°C for 10 s, and 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s.

LAMP primer designing

LAMP primers will be designed and used based on the 16srRNA gene. First, 16srRNA gene retrieved from Gene Bank (<http://www.ncbi.nlm.nih.gov/genbank/>) and *ClustalX* software was used for multiple sequence alignment and identifying conserved sequences. *PrimerExplorer Version 4* software (<http://www.primerexplorer.jp/e/>) was used for designing of all primers. Lastly, all primers were analyzed by BLAST (<http://www.blast.ncbi.nlm.nih.gov/>).

LAMP assay

Three sets of LAMP primers were designed from conserved and variable regions of 16S rRNA (Table 1). The master mix in a final volume of 25 µL containing 0.2 µM of each of F3 and B3 primers, 1.6 µM of each of FIP and BIP primers, 0.4 µM of each of LF and LB primers, 1X buffer, 1 µL Betaine (Sigma, St. Louis, MO, USA), 8 mM Mgso4, 1.4 mM dNTP, 3 µL DNA template and 8 units of Bst DNA polymerase (New England Biolabs, USA).

The temperature cycle was as follows: 3 minutes at 95°C, 90 minutes at 70°C, and 2 minutes at 96°C. Finally, results of LAMP were observed by the naked eye, under UV light, or after gel electrophoresis in 2% agarose gels stained with ethidium bromide (0.5 µg/ml). The length of LAMP products was compared to Gene Ruler 100-bp DNA Ladder Plus (Fermentas, Glen Burnie, MD, USA).

The sensitivity and specificity of LAMP

LAMP assay specificity using *M. hominis* standard strain PG21 (ATCC23114) and other microorganisms included *E. coli*, *Campylobacter* spp, *Klebsiella pneumonia*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Salmonella enterica*. All species were fully identified using standard biochemical and microbiological tests.

Analysis of the findings

After data collection, the specificity, sensitivity, NPV, and PPV of the LAMP and qPCR assays were compared to those of the conventional culture method, which provided the gold standard. The Kappa coefficient test was used for data analysis, using SPSS 21 software, and the results were reported at the significance level of 0.05.

Results

Out of 80 vaginal samples were collected in this study. The ages of the patients were between 21 to 65 years with a mean age of 41.2 years. The most common features in the study subjects were vaginal discharge (73.75%), vaginal itching, and burning (47.5%). For the evaluation of the LAMP assay, results were compared to those of the qPCR. As expected, the results showed that no positive signal occurred for any of the negative samples for both tests, confirming the specificity of the primer set

Table 1. The Primers used in Real-time PCR and LAMP assay

Primers	Sequence (5'-3')	Reference
MH 16S rRNA- F	TTTGGTCAAGTCCTGCAACGA	Pascual <i>et al.</i> , 2010
MH 16S r RNA-R	CCCCACCTTCCTCCCAGTTA	
TaqMan probe-VIC MH 16S rRNA	TACTAACATTAAGTTGAGGACTCTA	
F3	CCCACGTTCTCGTAGGGATA	This study
B3	GTTCGGATTGGAGTCTGCA	
LF	GCCTAAGGTAGGACTGGTGAC	
LB	TCACCGCAGCATAGCTGA	This study
FIP	GGTTTGCTAACCTCGGAGGCG-CTTGTTACGACTTCACCCCA	
BIP	GTGTGACGGGCGGTGTGTAC-CGGAATCGCTAGTAATCGCA	

for *M. hominis*. Among a total of 80 swab samples, 13 (16.3%) were positive by culture and 67 (83.7%) were negative.

The fourteen (17.5%) samples were positive for qPCR and 66 (82.5%) were negative. The LAMP assay was able to detect *M. hominis* in 25 of 80 vaginal swabs (Fig. 1, Fig. 2). Therefore, the LAMP technique was found to be highly sensitive than qPCR with a sensitivity of 100% (95% CI) and 46.2% (95% CI), respectively. Sensitivity, specificity as well as the PPV and NPV of LAMP and qPCR are shown in Table 2.

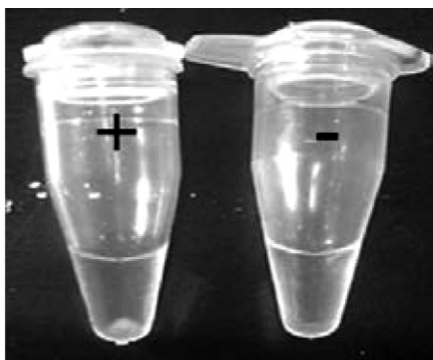


Fig. 1. Positive and negative reaction of the LAMP assay

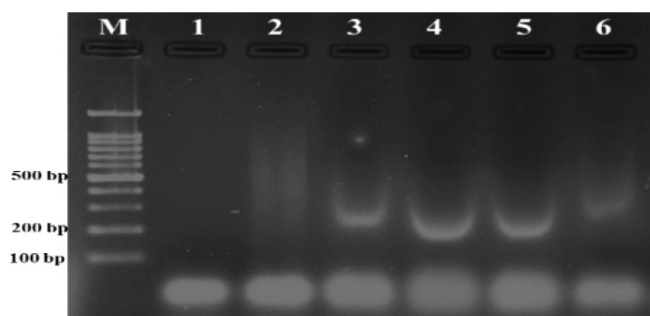


Fig. 2. Image of electrophoresis of LAMP products on agarose gel 2%. Lane M: 100bp DNA size marker (Fermentans, Lithuania), Lane (1): Negative sample, Lane (2-6): positive samples

The sensitivity of LAMP assays and qPCR was determined in terms of colony-forming unit (CFU) by making 10-fold serial dilutions of purified DNA from *M. hominis*. qPCR was able to detect up to the level of 10^4 CFU/ml, while LAMP could detect up to 10^3 CFU/ml. consequently, the detection sensitivity of LAMP assays was ten times more sensitive than the qPCR. The NPV of the LAMP assay was higher than the qPCR (100% vs. 89.4%).

Discussion

M. hominis, is one of the etiologic agents of nongonococcal urethritis (NGU) in women (Pascual *et al.*, 2010). *M. hominis* was the third most usual species among genital *Mycoplasmas* after *U. urealyticum* and *M. genitalium*. prevalence of *M. hominis* using both qPCR and culture methods was reported as 9.7%, in which 10.8% and 14% were among infertile women and men, respectively. Also, the incidence of genital *Mycoplasma* was higher in the central provinces of Iran, which was attributed to the lack of specialized STD clinics and infertility centers (Moridi *et al.*, 2020). Although culture is defined as a gold standard method for the diagnosis of *M. hominis*, high cost, the need for a rich culture medium, and time-consuming, as well as the need for skills a technician has limited its use in the laboratory. According to our results, *M. hominis* was more detected in women with 30-41 age than others. This result is the same as Zheng *et al.* (2020) study, which was attributed to the immune system status, childbearing period, and sexual activity of women in this range of age. The results show that the use of 16srRNA LAMP assay can be more effective in the diagnosis of *M. hominis* when compared to both culture and qPCR. The frequency of *M. hominis* was 31.3%, 17.5%, and 16.3% using LAMP, qPCR, and culture, respectively, in which one of the negative results was diagnosed by qPCR, while the LAMP assay detected twelve positives among the negative result of culture. This proved the high sensitivity of the lamp in identifying *M. hominis*. The false negative results of the culture as a gold standard method may be related to the storage and transfer condition to the laboratory which leads to a reduction in the number of bacteria (Bakhtiari *et al.*, 2019). Also, Baczynska *et al.* (2004) indicated the more effective impact of qPCR compared to culture on the diagnosis of *M. hominis* which mentioned it as an alternative assay over culture. In this study, LAMP assay was more specific and sensitive than both qPCR and culture. This can be explained by the fact that bacterial RNA is unstable and degrades rapidly in the environment, so the LAMP method is more effective compared to qPCR where mRNA is used. However, this prob-

Table 2. Evaluation of LAMP and PCR assay for the detection of *M. hominis* in vaginal samples

Results Tests	Positive No (%)	Negative No (%)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (%)	NPV (%)	Accuracy
RT-PCR	14 (17.5)	66 (82.5)	46.2	88.1	44.9	89.4	81.3
LAMP	25 (31.3)	55 (68.7)	100	83.33	56	100	86.3

lem can be solved by increasing the number of cells and then increasing the mRNA production of the desired gene, it's a complex process (Zhang *et al.*, 2011). This is also supported by Wang *et al.* (2018), which stated LAMP is useful in the diagnosis of genital *Mycoplasmas*, especially in cases where crude DNA is present. Bakhtiari *et al.* (2016) study, which set up the LAMP for diagnosis of *Helicobacter pylori* using a specific sequence of the *Ure C* gene, concluded that LAMP is a simple and rapid assay and has specificity and sensitivity. Edward *et al.* (2015) the LAMP assay was designed to detect *Mycoplasma genitalium* using the *pdhD* gene. They reported the sensitivity of the LAMP as similar to the sensitivity of the PCR method using the 16sr-RNA gene. However, LAMP assay-related disadvantages were reported as lack of true multiplexing capability and contamination risk due to high yielded amplicon (Karthik *et al.*, 2014), some advantages defined for LAMP over PCR based methods. LAMP assay three pairs of primers are used to amplify a sequence of gene, while in the PCR method only single forward and reverse primer is sufficient. Also, the time required for the lamp assay is half the time required to perform PCR assay (1.5 vs 3-4 hours). Other advantages including the high cost of equipment and complex PCR protocol, which is a limitation for PCR as a very specific method. In fact, instead of a thermocycler that requires different temperature adjustments, the LAMP assay only needs a water bath that is in all stages of an isothermal condition, as well as a single pipette (Njiru *et al.*, 2008).

Conclusion

According to the results, we established a rapid and sensitive LAMP method, which was applicable for detect *M. hominis* in vaginal swabs. So, the LAMP assay is a practical alternative for early detection.

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