

Detection of *Mycoplasma hominis* and *Ureaplasma Urealyticum* in urethritis from sexual active men by Real-time PCR

Haider A. Muhammid¹ , Habeeb S. Naher² , Adnan H. AL-Hamadani³

¹Department of Microbiology, College of Vet.Medicine ,Karbella University Iraq,

²Department of Microbiology, College of Medicine, Babylon UniversityIraq,

³Department of Microbiology, College of Medicine, AL-Qadisyya University Iraq

E.mail: hayder_1234@hotmail.com

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الخلاصة: تعد المايكوبلازما واليوريبلازما كائنات مجهرية فريدة عن بقية المايكروبات من خلال عدم امتلاكها للجدار الخلوي وهذه الكائنات المجهرية تكون داخلية التطفل تسبب اصابات بولية تناسلية ومن الصعوبة تتميتها على اوساط زرعية روتينية بسبب التعقيد في متطلبات الغذائية لذلك تعد تقنية تفاعل سلسلة البلمرة بالوقت الحقيقي ضرورية للكشف وتوصيف هذه الكائنات بسبب اقتصارها للزمن والكلفة والدقة والخصوصية والنوعية

هدف الدراسة: للكشف والتفريق بين المايكوبلازما واليوريبلازما المعزولة من المصابين بالتهاب الاحليل باستخدام تقنية سلسلة البلمرة في الوقت الحقيقي.

طرائق العمل: جمعت عينات احليل من 60 رجال مصابين بالتهاب الاحليل وكانت اعمارهم تتراوح بين (18-41) للفترة من كانون الاول 2012 الى ايار 2013 الذين راجعوا مستشفى الديوانية التعليمي العام وفحصت بتقنية تفاعل سلسلة البلمرة في الوقت الحقيقي للكشف عن المايكوبلازما التناسلية. **النتائج:** من مجموع (60) عينة، 28 عينة كانت موجبة للفحص وكالاتي : 3 (10.7%) للمايكوبلازما كعينة فريدة ، 17 (60.7%) لليوريبلازما كعينة فريدة و 5 (17.8%) عينة مختلطة لكلا النوعين.

الاستنتاج: اظهر فحص سلسلة البوليمريز في الوقت الحقيقي دقة عالية في تشخيص والتفريق بين انواع المايكوبلازما من العينات السريرية.

Background: *Mycoplasma spp* and *Ureaplasma spp* are unique microbes in they lack a cell wall and cause genitourinary tract infections ,these organisms are considered an intracellular parasites and difficult to cultivate in routine media due to the complexity of their nutritional requirements so , the molecular detection and identify these microorganisms by Real-Time PCR due to cost, time, reliability, specificity, and sensitivity.

Aim of study : To detect and differentiate *Mycoplasma hominis* and *Ureaplasma urealyticum* isolates from patients with urethritis by Real-Time PCR.

Methods: Urethral swabs collected from 60 sexual active men from December 2012 to May 2013 who attending to AL- Diwanyia Teaching Hospital were tested by using DUPLICa RealTime PCR kit for the presence the of genital mycoplasmas (*Mycoplasma hominis* based on amplification of 16SrRNA gene and *Ureaplsma urealyticum* based on amplification of *urease gene*) , the age of patients study with urethritis were (18-41) year old.

Results: Out of 60 Urethral samples there 28 were positive samples as 3 (10.7%) , 17 (60.7%) and 5 (17.8%) for *M.hominis* (as a single pathogen) ,*U.urealyticum* (as a single pathogen) an both of genital mycoplasma respectively the age group (30-35) years old was more significant (P<0.05) than other group ages

Conclusion : Real-PCR shows a rapid and high accuracy in detection and differentiation of *M. hominis* and *U. urealyticum* from clinical samples. **Keywords:** Mycoplasma , Ureaplasma , Real-Time PCR , Urethritis

Introduction

Mycoplasmatales are associated with infections of the genitourinary tract, reproductive failure and neonatal morbidity and mortality. (1,2) Genital Mycoplasma has also been associated with pelvic inflammatory disease (PID), endometritis, arthritis and cervicitis. (3) *Ureaplasma urealyticum* is the main cause of non-gonococcal, non-chlamydial urethritis, acute prostatitis and acquired arthritis in men. In pregnant and non-pregnant women, Ureaplasma can cause chorioamnionitis and pre-term delivery, abortion, pre-term birth, vaginitis and cervicitis. (4,5) Clinical studies have demonstrated that infants born to infected mothers become infected with these bacteria, and colonization of the respiratory tract of infants has been associated with pneumonia, respiratory distress and meningitis(3). Urethritis, which is one of the most common sexually transmitted diseases in men, is classified as gonococcal or non-gonococcal depending on the presence or absence of *Neisseria gonorrhoeae*. *Chlamydia trachomatis* is a cause of acute non-gonococcal urethritis (6).

Real-time PCR technology has recently been exploited in clinical microbiology. It is a promising tool with an excellent sensitivity and specificity, very fast and suitable for high throughput of samples with an inherent quantitative ability therefore the conventional PCR provided limited applications for bacterial diagnostics due to the technical difficulties required for performing the procedure and the time delay in producing a final result , moreover certain specimens such as sputum , urethral and feces were difficult to test due to interinsinc substances that inhibited PCR chemistry and therefore the sensitivity of the assay

was significantly compromised , as a result conventional PCR testing methods were limited to bacteria that are difficult to culture or grow slowly (e.g., *Legionella spp.*, *Mycoplasma spp.*, *Chlamydia pneumoniae* , *Bordetella pertussis*)(7).

According to knowledge's clinical studies on the organisms and their role in colonization of human urogenital Mycoplasma in Iraq population are rare except the study of (8) whom success in the cultivation of mycoplasmas isolates from bacterial vaginosis, therefore the aim of this study is Identifiy of genital Mycoplasma (*M.hominis* and *U.urealyticum*) in sexual active men suffering from urithritis in AL-Diwania city Iraq.

MATERIALS AND METHODS:

Clinical Specimen : From December 2012 to April 2013, 60 Individuals admitted to the hospital for a general checkup participated voluntarily and a brief questionnaire was obtained. Sexually active, asymptomatic people aged between 18 and 41 years were enrolled. We defined 'sexually active' as having had sexual intercourse in the preceding 3 months. People who had a recently cured genitourinary infectious disease within 3 months were excluded. People with genitourinary symptoms such as pyuria , dysuria, or itching in the genital area were also excluded. Finally, 60 persons were assigned to be tested. Swabs were placed in Mycoplasma transport medium (LioFilChem ,Italy) to maintain the swabs wet and directly transported to the lab for bacteriological investigation (4) .

Pretreatment of urethral specimens and DNA extraction:

The specimens was equilibrated to room temperature and centrifuged at 5000xg for 15 minutes. The supernatant

was discarded, and the pellet was resuspended in 1 ml 1xPBS before DNA extraction. Genomic DNA was extracted from the pretreated specimens (swab or urine) by using the QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real time PCR reaction assay:

DUPLICα RealTime PCR detection kit for *Mycoplasma hominis* and *Ureaplasma Urealyticum*, which were

originally designed according to manufacture processes, were chosen from the (Euroclone serving science innovation Germany) of the conserved intergenic spacer region in the 16S rRNA gene of the *Mycoplasma hominis* and urease gene for *Ureaplasma urealyticum* were used in order to detect *M. Hominis* and *U. urealyticum* simultaneously (Table 1).

Table (1): Compositions of DUPLICα RealTime PCR detection kit for *Mycoplasma hominis* and *Ureaplasma Urealyticum*.

Time	Temperature	Cycle
10 minute	95°C	1
15 second	95°C	50
60 second	60 °C <i>Ureaplasma urealyticum</i> 52 °C <i>Mycoplasma hominis</i>	

Reagents	Composition
Amplification mix	Hot star taq DNA polymerase Nucleotides, MgCl ₂ , e buffer
Oligo Mix	Specific primer, Fluorogenic probes
Internal positive control of amplification	Specific DNA with Specific primer
Reaction Blank	-DNA for genital mycoplasma

In the new sterile premix PCR tube were prepared 25 µl of RT-PCR mix, the reagent of the mix have to mixed under this ratio. Once the mix is ready, aliquot 21ul of Master Mix in the tubes for RT-PCR and add in each tube 4ul of extracted DNA and of controls, After that, RT-PCR PreMix were added into PCR PreMix tube. Then, real-time PCR tubes sealed by

the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes centrifuge at 3,000 rpm, for 2 min , then start Exicycler™ 96 Real-Time Thermal Block , the tubes were set inside the instrument and started the program of amplification according to kit instruction (Table 2):

Table (2) Thermal profile for *M.hominis* and *U.urealyticum*:

Statistical analysis

The Statistical package for social sciences (SPSS) version 11 test was applied to calculate LSD (least significant different) for categorical data(9).

Of the 60 sexual active men studied there were 28 samples were positive and expressed as following 3(10.7%) , 17(60.7%) and 5(17.8) were positive for *M. hominis* (as asingle pathogen) , *U.urealyticum* (as asingle pathogen) and both of genital mycoplasma respectively (Table 3) Figure (1,2).

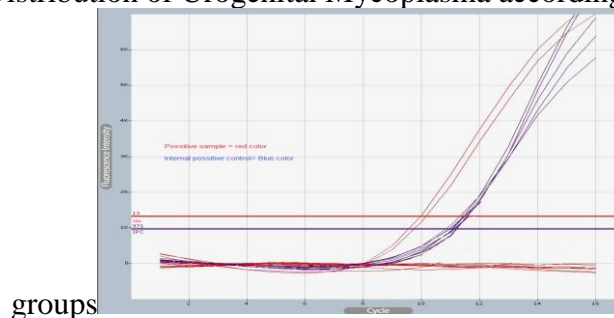
Results and Discussion:

Table 3:Percentage of Urogenital Mycoplasma isolated from urethral swabs by using Real-Time PCR technique

Urogenital Mycoplasma	RT-PCR	Negative results
<i>M. hominis</i>	3(10.7%)	57
<i>U.urealyticum</i>	17(60.7%)	43
Both	5(17.8)	55
Total 60	28	32

The age of the patients from who were PCR positive was varied from 18-41 with a mean age of 24.5 years, distribution of the genital mycoplasmas in accordance to patient's age showed that the age group in 30-35 years old was more significant ($P<0.05$) than other aged group (Table 4).

Table 4: Distribution of Urogenital Mycoplasma according to the aged



Age group in years	<i>M. hominis</i> NO.	<i>U.urealyticum</i> NO.	Mixed isolates NO.
18-23	0	2	1
24-29	1	4	2
30-35	2	9	4
36-41	0	2	1
Total 28	3	17	8
LSD	0.9414	2.4908	1.3314

Figure 1: Amplification curve of DNA extraction of *Mycoplasma hominis* by using Real-Time PCR

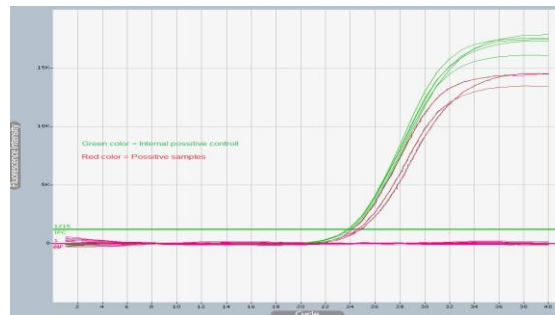


Figure 2: Amplification curve of DNA extraction of *Ureaplasma Urealyticum* by using Real-Time PCR

In the present study, 46.6% of 60 active sexual men with Genital mycoplasmas as detected by RT-PCR. *U. Urealyticum* (60.7% in 28) has been shown to be more frequently detected in men than *M. hominis* (10.7%). Other studies using PCR for detecting Genital mycoplasmas in endocervical specimens have reported prevalence rate as high as 40 to 80% for *U. urealyticum* and 20-50% for *M. hominis* (10,11). Our results agree with these studies which differences were not statistically significant, but the isolation rate of genital mycoplasmas were higher in men in 30-35 years of age that is consistent with that previously described by others (11) whom stated that ages are more active sexually. RT-PCR amplification was performed with the of DUPLIC α RealTime PCR detection kit for

mycoplasma hominis and DUPLIC α RealTime PCR detection kit for *Ureaplasma Urealyticum* , according to the manufacturer's instructions. The kit allows to find out Ureogenital Mycoplasma DNA by using specific fluorogenic sequence probes. The fluorogenic probes are made of an oligonucleic sequence which presents at its 5'-end a fluorescent dye called (reporter), while at the 3'-end there is a second dye called (quencher), The test can detect the specific product of amplification by monitoring the increase of the fluorescence signal which is proportional to the quantity of the product amplified; the high specificity of the system allows the probe to choose between fragments which differ from only one nucleotide(13). We checked for present

of absent of amplification by genes which encoded for *M.hominis* 16SrRNA gene and Urease gene which encodes for *U.urealyticum* according to the kits was taken advantage of an Oligo Mix containing a Mycoplasma hominis specific probes and *Ureaplasma urealyticum* specific probes linked at its 5'-end to a FAM molecule (6-carboxyfluorescein). In addition to this, the Oligo Mix contains internal control (IC) specific probes, linked at its 5'-end to a HEX molecule (hexa-chloro-fluoresceine), the two probes have at their 3'-ends a quencher called (BHQ-1). An internal control was included in the RT-

PCR mix to detect the presence of PCR inhibitors, for the negative control, sterile deionized water was used as the PCR template instead of nucleic acid, the positive control contained in the kit was assayed to check the integrity of the primers used in the RT-PCR assay (11) , Our study resemble (14, 15) that used yidC probe and primer set in Real-time PCR for detected *M.hominis* , and *mg219 gene* of Real-time PCR for detected *M.genitalium* respectively. In conclusion , the present data proved that molecular detection is a useful tool for diagnosis the mycoplasma spp. In clinical specim

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