KUFA JOURNAL FOR NURSING SCIENCES Vol.5 No. 2, May through August 2015

Using Multiplex PCR Assay for Detection of Genital Mycoplasmasand Ureaplasmaspp in an Infertility Males.

استخدام تقنية سلسلة تفاعل الانزيم البلمرة في تحديد المايكوبلازما واليوريابلازما عند الرجال العقيمين

Ihasan A. H. Al-Talqani\ Baghdad of University \ B.Sc. In Medical and Healthy Technique.
Dr. Habeeb S. N. Al-Musawi\ Prof. University of Babylon\ College of Medicine.
Ahasan.iraq@gmail.com

الخلاصة:
الهدف : تهدف الدراسة إلى تشخيص الأنواع البكتيرية يوريابلازما و مايكوبلازما من خلال التحري عن جينات multiple banding antigen gene(MBA)و 16sRNAعلى التوالي في عينات السائل النوي باستخدام تقنية تفاعل لأنزيم البلمرة المتعدد (Multiplex-PCR).
المنهجية : تضمنت الدراسة ، جمع (106) عينة سائل منوي ، تم جمع (86) من رجال عقيمين تم تشخيصهم من قبل اطباء اختصاص في العقم و (20) نموذجا من رجال اصحاء كنماذج سيطرة خلال ستة اشهر (تشرين الثاني 2013 ولغاية نيسان 2014) . تم تحليل النتائج باستخدام الوسائل الإحصائية (T-test) spss . تم الحصول على جميع هذه العينات من الاشخاص الذين حضروا إلى مركز العقم في مدينة الصدر الطبية في محافظة النجف.
النتائج : كشفت نتائج تقنية سلسلة التفاعل لأنزيم البلمرة المتعدد (multiplex-PCR) من 86 عينة وجدت13(% 15.1) نتيجة موجبة من رجال عقيمين تضمنت *M.hominis*5(5.8%)، *U.urealyticum* 5(5.8 %) و *U. parvum* 3(3.5%).
الاستنتاجات : هذه الدراسة تستنتج ان تقنية سلسلة التفاعل لأنزيم البلمرة المتعدد كفاءة كبيرة وسريعة في تشخيص جنس اليوريابلازما والمايكوبلازما في السائل النوي.
التوصيات : دراسة تحديد انواع اخرى من الاحياء المجهرية (الفيروسات، الفطريات ،كلاميديا والسيلان النيسيري).

ABSTACT
Objective: This study aim to diagnosis the types of bacteria*Ureaplasmaspp*and *Mycoplasma hominis* by screening for multiple banding antigen (MBA) gene and 16S rRNA respectively in seminal fluid by using the multiplex-PCR technique.
Methodology: The study included , a total (106) semen fluid samples, were collected from 86 infertile men which have been diagnosed by specialized clinicians specialization in infertility and 20 other semen fluid samples from healthy men used as control through six months (November 2013 until April 2014) . All these Date was analyzed by using spss(T-test) program All these samples were obtained from subjects who attended to center infertility in medical city at Al-Sadar in Najaf province.
Results: The result of the multiplex-PCR revealed positive results in 13(15.1%) of the 86 seminal fluid samples from infertile patients which represented *M.hominis* 5(5.8%), *U.urealyticum* in 5(5.8%) and *U.parvum*3(3.5%).
Conclusion: in light of the results obtained this study we concludes that the efficiency of multiplex-PCR large and rapid in diagnosis of *Ureaplasmaspp* and *Mycoplasma hominis* in seminal fluid.
Recommendation : the study of detection of other microorganism (viruses, fungi, *Chlamydia* and *Neisseria gonorrhea*) in men infertility.
Key words: Multiplex-PCR,*Mycoplasma, Ureaplasma*,Men, Infertility.

INTRODUCTION

Mycoplasmas and *ureaplasmas*, belonging to the family *Mycoplasmataceae*and *Mollicutes*class, are widely distributed in humans, mammals, birds, reptiles, fish, and other vertebrates as well as in plants [(1)].They are the smallest free living organism known on the planet able to multiply autonomously [(2)].*Mycoplasms* do not have cell-wall, so can take many different forms by the absence of precursors of peptidoglycan which make them difficult to identify. There is an attachment organelle at the tip of filamentous. Fried –egg-shaped colonies are seen on agar and have a double-stranded DNA and divide by binary fission. Most interesting is the use of the universal stop codon UGA as tryptophan codon. It is so difficult to cultivate them in the laboratory and are often missed as pathogenic causes of disease [(3)].*Mycoplasmas* usually require cholesterol for growth and membrane function and are filterable through the usual bacteriological filters**.** Resistance of Mycoplasmal RNA

polymerase to rifampicin is another property distinguishing mycoplasmas from the conventional bacteriaMycoplasmasconstitute a large group of microorganisms but only a few species, i. e. *Mycoplasma* and *Ureaplasma*species, are pathogenic for humans, where they mainly inhabit the mucous membranes of the respiratory tract and genitourinary system,Three species have been isolated from the mucosal membrane of the genitourinary tract: *Mycoplasma hominis, Ureaplasmaurealyticum* and recently discovered *Mycoplasma genitalium*(4).The role of mycoplasmas in the etiopathogenesis of inflammatory states of the genitourinary organ is still a subject of controversy.Their presence has been associated with non-gonococcal urethritis, vaginitis,cervicitis, pelvic inflammatory disease(PID))and pathology of pregnancy and newborns. *M. hominis* has been reported in 58-76% of women with bacterialvaginosis(BV) and is the only genital mycoplasma which is consistently more oftenisolated from vaginal swabs in women with(BV) than those without BV(5)**.**Genital mycoplasmas and ureaplasmasseem to be widespreadamong the male partners of infertile couples in Iraq, The aim of this study is to determine of *Mycoplasma hominis*and*Ureaplasmaspp*from infertile men in Al-Najaf province by using duplex primer assays.

MATERIAL AND METHOD

Sampling:

A total at 106 semen fluid samples were collected from 86 infertile men and 20 other semen fluid samples from healthy men used as control. All these samples were obtained from subjects who attended to center infertility in medical city at Al-sader Hospital in Najaf province through a period at 6 months (from November 2013 to April 2014). Swabs were inserted in tubes containing special transport medium to maintain the swabs. Each specimen was coated in ice bag until be taken to the laboratory for bacteriological analysis. Specimens were first incubated at 370C for 30 minutes, then a loop-full from each sample was transferred to transport media (H-I broth)according to(6)for detection the *Mycoplasma hominis, Ureaplasmaurealyticum*and *ureaplasmaparvum* respectively(7,8). After incubated this tube in anaerobic jar at 37^0C for 24-72 hours in the laboratory

Molecular assay: Molecular experiments included the extraction and amplification of *Mycoplasma* and *Ureaplasma spp.* DNA. for extracted DNA by G-spin ^{TM} total DNA extraction kit (intron)according manufacture company. Multiplex PCR was performed with primers specific for highly conserved regions in the multiple band antigen(MBA) gene of Ureaplasma spp and the 16S rRNA gene of *M. hominis*(9,10)show in table(1). Preparation of primers suspension the DNA primers were resuspended by dissolving the lyophilized primers provided by (Integrated DNA Technologyies -USA) after spinning down with TBE buffer depending on manufacturer instruction as stock suspension as recommended by(11,12)show in table (1).

Table(1). Nucleotide sequences of multiplex primer

Organism	Primer	Sequence (5'-3')	Size of amplified product(bp)	Target gene
M. hominis	**RNAH1** **RNAH2**	**CAATGGCTAATGCCGGATACGC** **GGTACCGTCAGTCTGCAAT**	**334**	**16SrRNA**
U.urealyticum	**UMS125** **UMA226**	**GTATTTGCAATCTTTATATGTTTTCG** **CAGCTGATGTAAGTGCAGCATTAAAT TC**	**403 or 448**	**MB antigen gene**

The reaction mixture: Amplification of DNA was carried out in a final volume of 25 µl containing the contents according(9).All reactions were performed in a Gene amplification PCR System 9600 Thermocycler (Perkin-Elmer, Norwalk, Conn.) under the following

conditions: 1 cycle of 10 min at 95°C, followed by 35, two-step cycles of 95°C for 15 s and 60°C for 60 s, followed by 5 min at 72°C.

Detection *M. hominis*and *U .urealyticum*by Multtiplex-PCR: Amplification of specific gene 16S rRNA for *M. hominis*and multiple banding antigene (MBA) gene for *U. urealyticum* made according to method recommended by [13]show in table (1) .

Detection of amplified products by agarose gel electrophoresis: Successful PCR amplification was confirmed by agarose gel electrophoresis as mentioned by [14].Agarose gel was prepared by dissolving 2 gm of agarose powder in 100 ml of TBE buffer 1X(pH:8) in boiling water bath, allowed to cool to 45^{o}C and ethidium bromide at the concentration of 0.5mg/ml was added. The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, and 10µl of each DNA sample was transferred into the wells in agarose gel, and in one well we put the 10µl of DNA ladder. The electric current was allowed at 65 volt for 90 min. UV transilluminater was used for the observation of DNA bands, and gel was photographed using a digital camera.

Statistical analysis

T-Test recommended by [15] was used for statistical analysis to show if there is any significant differences between results.

RESULTS :

The PCR amplified assay revealed positive results in 13(15.1%) of the 86 semen samples from infertile patients which represented *M. hominis*5 (5.8%) samples, *U.urealyticum*in 5(5.8%) samples and *U.parvum*3(3.5%) as shown in table (2).

Table(2) percentage of isolated mycoplasmas by using multiplex PCR

	Bacterial species	Single isolates *(n)*	Mixed isolates *(n)*	Total isolates *(n)* (%)	Product Size
Bacteria detection by multiplex PCR	*Mycoplasma hominis*	4	1*	5(5.8%)	334bp
	Ureaplasmaurealyticum	4	1*	5(5.8%)	448bp
	Ureaplasmapavum	1	2*	3(3.5%)	403bp
Total		9	4	13(15.1%)	

Table (2) shows that two isolates of *U.pavum* were mixed with one isolate of *U.urealyticum* and one isolated *M.hominis.*

Table(3)Means ± standard deviations (SD) parameter semen of considered variables in each group of patients.

Bacteria / semen Parameter	M. hominis	U.Urealyticum	U.Parvum	Control
Sperm count	21.0±25.3***	51.80±56.6**	35.0±49.4**	85.±11.8
Agglutination	6.0±9.29***	90.0±13.41***	5.0±7.07***	0
Leukocyte	17.0±8.45***	23.80±13.7***	25.0±7.07**	0
Motile	8.67±10.32	17.0±21.67***	5.0±7.07***	68.95±5.08
Sluggish	10.4±8.64*	13.0±17.17*	10.0±14.14*	14.30±6.91
Immotile	47.3±37.3***	50.0±38.56**	35.0±49.44*	16.75±6.83
Normal	17.8±14.6***	30.80±24.8***	7.5±70.6***	95.55±4.47
Abnormal	48.8±38.2***	49.2±32.19***	42.5±60.1**	4.45±4.47

*P<0.05, **P<0.01, ***P<0.005.

This table explain the different variables in each group with those of the control group (20 fertile individuals). The number of patients considered for each group is reported in parentheses.

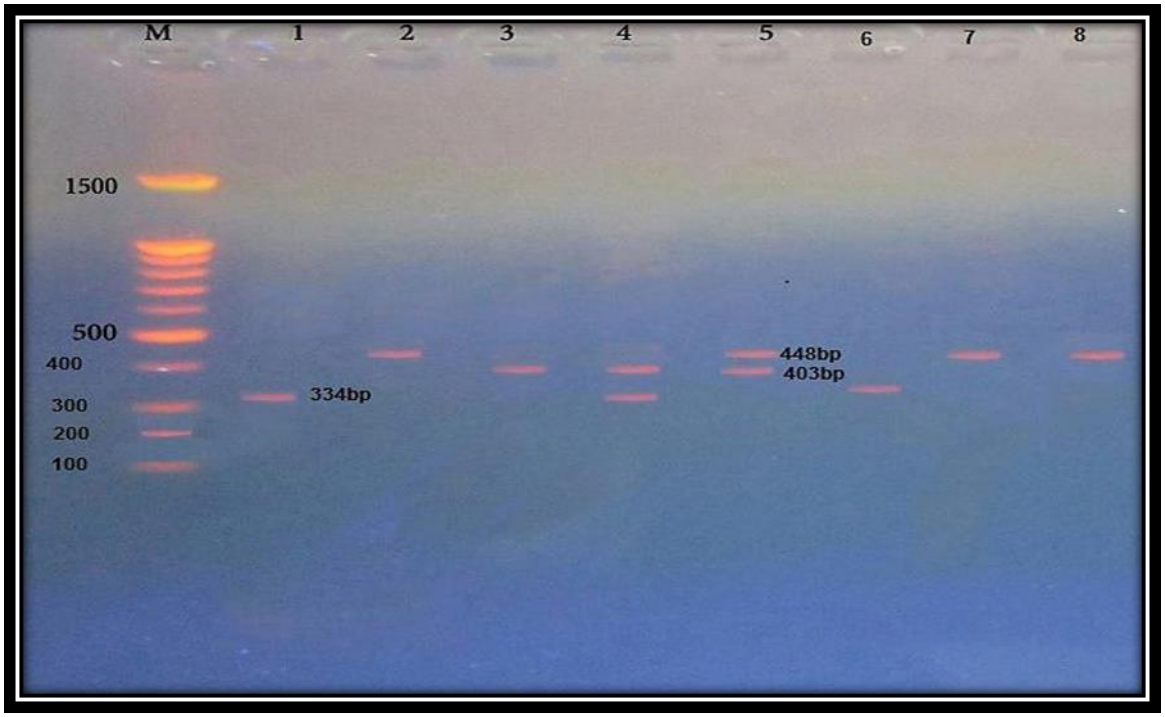

Figure (1) A photograph of Ethidium bromide stained by 2% agarose gel showing in gel electrophoresis for multiplex PCR product (334bp-*M.hominis*-403-*U.parvum*,448bp-*U.urealyticum*,)

Figure(1) Show M: 100 bp standard size reference marker. Lane 1, lane 4 and lane 6, *M.hominis* positive results, Lane 2, lane 5and lane 7 and lane 8 *Ureaplasmaurealyticum* positive results and Lane 3, lane 4 and lane 5 *Ureaplasmaparvum* positive results. Lane 4 are mixed between *M.hominis and Ureaplasmaparvum* and lane 5 are mixed between *Ureaplasmaurealyticum* and *Ureaplasmaparvum*.

DISCUSSION

Other fastidious, non-cultivable bacteria are also included in this study using the multiplex polymerase chain reaction (Multiplex-PCR) technique for the detection of these bacteria accordingly *Mycoplasma hominis* represents, *Ureaplasmaurealyticum* and *Ureaplasmapavum*.

However, this reliability is limited by PCR technique which has the advantages of being easy and rapid in detection these organisms, but the matter is, there are no commercially available molecular tests, therefore, efforts for new techniques to detect *Mycoplasma* and *Ureaplasma* are ongoing at present time since various procedures based on DNA amplification have been developed for detection of M. pneumonia.

Various phenotypic and molecular methods have been described by other research to distinguish the two main groups of human urea plasmas (formerly two biovars of *U. urealyticum*, now proposed species *U. parvum* and *U. urealyticum*). Our results showed that there is a homology between sequences of the multiple binding antigen genes(MBA) subunits of biovars within species was used subtyping two biovars by using universal primer which distinguished between them depending on the specific product 403bp for *U. parvum* and 448 bp for *U. urealyticum* and 338bp for *M.hominis* that show in figure (1). Accordingly, it can be concluded that the results be achieved by this study, can be a confirmation, for other studies[14].

The comparison of semen characteristics between infected and non-infected men showed that motile spermatozoa and viability were lower when present the genital Mycoplasma and genital urea plasma. *M. hominis* could cause extra genital and systemic infections in people suffering from immunodeficiency syndromes [16].Studies in human reproduction and *in vitro* fertilization showed that *M. hominis* adheres to or invades the human sperm cells, showing no apparent damage and significant effects on sperm count, motility and fertilization**.** With respect to women, mycoplasmas cause infertility by impairing normal sperm function in the cervix and causing endometritis and tubal damage. M.hominis invades human sperms and result in non- apparent or subtle damage and might have implication for long-term infertility [17].*U. urealyticum* can adhere to the sperm membrane ,thereby potentially causing gamete dysfunction and enhance the adverse effects of superoxide and hydrogen peroxide produced by the organism, with subsequent spermatozoan hyper production of reactive oxygen species(ROS)[18]. The ROS induce lipid peroxidation ,which reduces membrane fluidity and sperm fertilization capability, and may be the mechanism by which *U.urealyticum* impairs sperm function[19]. Other works have reported that the presence of *U. urealyticum*in semen was related to a decrease in sperm concentration [20] Show in table (2).

Other study[21],have investigated the occurrence of *M. hominis*in first-voided urine specimens from young men. They found that the occurrence of *M. hominis* was 4%. They concluded that there is a need to use PCR to determine the of Mycoplasmas role in STDs. More over Yoshida and coworker, stated that U.urealyticum strongly associated with males urethritis by using PCR technique. Recently, PCR technique was successfully used in Iraq by our team work[22]. Other study the most common bacterial types isolated from patients with bacteriospermia were *C. trachomatis* (41.4%), *U. urealyticum*(15.5%) and *M. hominis*(10.3%) [23].

Enhanced sensitivity for genital Mycoplasma detection with PCR is consistent with the literature [24,25]. This finding is not surprising given the fact that mycoplasmas are labile organisms lacking a cell wall. PCR has an advantage in that it can still detect nonviable organisms.

Another advantage of the multiplex PCR is that the presence of other microorganisms does not interfere with testing. Indeed, the specimen excluded from study due to bacterial overgrowth was actually positive for *Ureaplasma* by PCR. Finally, the multiplex PCR is a relatively rapid assay that can be performed in less than 8 h. Isolation by culture may take 2 to 5 days to obtain a result for *Ureaplasma spp*. and *M. hominis* The rapid detection of genital mycoplasmas and Ureaplasma spp is very important, particularly in the management of pelvic inflammation disease, infertility, in whom these organisms are a significant cause of meningitis, respiratory disease, in and death.

CONCLUSION

Both, *Ureaplasmaspp* and *Mycoplasma hominis* are frequently infect genitourinary tract of men . *U. urearilyticum*seems to be more frequent. Multiplex- PCR assay is fully efficient in detection of genital mycoplasmas and/or *Ureaplasmasspp,* consequently, rapid detection, confirmed identification and reliable diagnosis of these organisms.

RECOMMENDATIONS:

1- Further study can be designed to detect the role of other organism (viruses, fungi, Chlamydia and Neisseria gonorrhea) in men infertility.
2- A achievement further study on Mycoplasma and Urea plasma to determine the exact effect on spermatozoa.

REFERENCES:

1. Yoshida T, Maeda S, Deguchi T, Ishiko H:(2002). Phylogeny-based Rapid identification of mycoplasmas and ureaplasmas from urethritis patients. ***J ClinMicrobiol*, 40:105-10.**
2. Lincoln, C.K.(2008).*Mycoplasma* testing, Bionque laboratories, htt: **www. Biunique. Com .(Abs).**
3. Pascual,A.;Jaton,K.;Ninet,B.;Bille,J.; and Greub,G.(2010). New Diagnostic Real –Time PCR for Specific Detection of *Mycoplasma hominis*DNA.J.**International Journal of Microbiology.10:1-4.**
4. Zdrodowska-Stefanow B, Kłosowska WM, Ostaszewska-Puchalska I, Bułhak-Kozioł V, Kotowicz B. (2006). Ureaplasmaurealyticum and Mycoplasma hominis infection in women with urogenital diseases. **Adv Med Sci. 51: 250-3**.
5. Uusküla A, Kohl PK. (2002). Genital mycoplasmas, including Mycoplasma genitalium, as sexually transmitted agents. **Int J STD AIDS.13: 79-85**.
6. Naher H.S., Said I.H. and Al-Hamadini A.H., Modified culture medium for isolation and identification of Mycoplasma hominis and Ureaplasmaurealyticum in Women in Al-Qadisiya Province In press (2013).
7. Razin S. and Herrmann R., (2002) Molecular Biology and Pathogenicity of Mycoplasmas. **Kluwer Academic/Plenum Publishers, New York**.
8. Golshani M, Eslami G, GhobadlooShMohhammadzadeh, Fallah F, Goudarzi H, Rahbar AA Soleimani, et al. Detection of Chlamydia trachomatis, Mycoplasma hominis and Ureaplasmaurealyticum by multiplex PCR in semen sample of infertile men. ***Iranian J Public Health.* 2007;36(2).**
9. Yoon, B. H., R. Romero, J. H. Lim, S. S. Shim, J. S. Hong, J. Y. Shim, and J. K. Jun. 2003. The clinical significance of detecting Ureaplasmaurealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. **Am. J. Obstet. Gynecol. 189:919–924.**

10. Blanchard, A., A. Yanez, K. Dybvig, H. L. Watson, G. Griffiths, and G. H.Cassell. 1993. Evaluation of intra species genetic variation within the 16SrRNA gene of Mycoplasma hominis and detection by polymerase chain reaction. **J. Clin. Microbiol. 31:1358–1361.**
11. Yoshida T, Maeda S, Deguchi T, Miyazawa T, Ishiko H. Rapid detection of Mycoplasma genitalium, Mycoplasma hominis, Ureaplasmaparvum, and Ureaplasmaurealyticum organisms in genitourinary samples by PCR-microtiter plate hybridization assay. ***J ClinMicrobiol.*** **2003;41(5):1850-5.**
12. Boesen T., Fedosova N.U., Kjeldgaard M., Berkelund S. and Gunna K. (2001). Molecular design of Mycoplasma hominisVaa adhesion, ***Protein Sci*****., 10, 2577-68.**
13. Stellrecht, K. A ; woron, A. M. ;Mishrik, N. G. and Venezia, R. A. (2004). Comparison of Multiplex PCR Assay with Culture for Detection of Genital Mycoplasmas **JOURNAL OF CLINICAL MICROBIOLOGY,24(4):1528-1533.**
14. Peerayeh S.N. and Samimi R., Comparison of Culture With The Polymerase Chain Reaction for Detection of Gennital Mycoplasma, ***Eur. J. Gen. Med*****., 5, 107-111 (2008).**
15. Danial, W. (1988). Biostatistics a foundation for analysis in health sciences, **4th ed. John Wilely and Sons, Inc.**
16. Diaz-Garcia F J, Herrera-Mendoza A P Giono-Cerezo S Guerre, Infanate F M (2006). *Mycoplasma hominis* attaches to and locates intracellularly in human spermatozoa. **Hum. Repro. 21:1591-1598.**
17. Potts ,J. M, Ward A M and R RRackley (2000). Association of chronic urinary symptoms in women and *Ureaplasmaurealyticum*. **Urology 55:486-489.**
18. Sandlow, J, L.(2004). Do varicoceles really effect male fertility, sexuality, reproduction and **menooause. 2:219-221.**
19. Wang Y, Liang CL, Wu JQ, Xu C, Qin SX, Gao ES: Do *Ureaplasmaurealyticum* infections in the genital tract affect semen quality?***Asian J Androl*****2006, 8:562-568.**
20. Takahashi, S., Takeyama, K., Miyamoto, S., Ichihara, K., Maedo, T., et al. (2006). Detection of *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasmaurealyticum*and *Ureaplasmaparvum*DNAs in urine from asymptomatic healthy young Japanese men.
21. Hayder N. Ayyez, Habeeb S. and Mohammed A. K.Alsaadi,(2014).Serological and Molecular detection of *Mycoplasma pneumonia*. **Int.J. Curr. Microbiol. App. Sci..3(9): 1201-1206.**
22. Gilmore MS. (2002)The *Enterococci*: pathogenesis, molecular biology, and antibiotic resistance. **Washington: ASM Press.**
23. Abele-Horn, M., C. Wolff, P. Dressel, A. Zimmermann, W. ahlensieck, F. Pfaff, and G. Ruckdeschel. (1996). Polymerase chain reaction versus culture for detection of *Ureaplasmaurealyticum*and *Mycoplasma hominis*in the urogenital tract of adults.
24. Johnson, E. T., T. A. Green, J. Schachter, and A. L. Et. (2000). Evaluation of nucleic acid amplification tests as reference tests for *Chlamydia trachomatis* infections in asymptomatic men. **J. Clin. Microbiol. 38:4382–4386.**
25. Teng, K., M. Li, W. Yu, H. Li, D. Shen, and D. Liu.(1994). Comparison of PCR with culture for detection of *Ureaplasmaurealyticum*in clinical samples from patients with urogenital infections. **J. Clin. Microbiol. 32:2232–2234**.