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Isolation and Identification of some *Mycoplasma* spp. from Urinary tract infection in Basrah City by Monophasic-Diphasic Culture Setup (MDCS) method and PCR

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Abstract

A total of Two hundred urine samples were collected from males and females who admitted toAl- Basrah general hospital,Al--Sadr teaching hospital, Al –Fayhaa general hospital and Al-Mawani general hospital in center of Basrah city with signs and symptoms suggestive of UTI for the period from 1st November 2012 to April 2013, The age of patients ranged from 5-65 years. Another 50 urine samples were collected from patients attending outpatient clinics without signs and symptoms of UTI and considered as control. In an attempt to comprised isolation and identification of Mycoplasma spp and detection of bacteria other than Mycoplasma, Culture by monophasic-diphasic culture setup (*MDCS) method(monophaasic-diphasic culture setup) and PCR techniques were used to detect Mycoplasma spp in genitourinary tract infections in this study. Culturing method basing on diagnostic characteristics revealed positive results of Mycoplasma spp in 87 cases(43.5%) included; Ureaplasma urealyticum in 73 cases (36.5%) while 13 cases (6.5%) were positive for M hominis and Mycoplasma fermentans in one case.(0.5%). 73 isolates of U. Urealyticum and 13 isolates of M. hominis were selected for PCR investigation. The results of this method indicated that 35/73 (47.9%) isolates of U. urealyticum were positive with PCR method versus 38/73(52%) were negative while among the 13 isolates of M. hominis, only 6/13 (46.1%) were positive with PCR technique versus 7/13 (53.8%) isolates exhibited negative results, culturing methods for diagnosis of bacteria are still* the bast method. *Escherichia coli* was the first causative agent of infection 73(36.5%) then *Staphylococcus aureus 40(20%), Klepsiella pneumoniae 25(12.5%)Pseudomonas aeruginosa 7(3.5%),Proteus2(1%) .*

Keywords: *Mycoplasma* spp., UTI,

Introduction

Mycoplasma are the smallest cell free-life microorganisms, characterized by their small cell size (0.3-0.8µm) and thus can pass through some filters used to remove bacteria. They have the smallest genome size and as a result, lack many metabolic pathways and lack of rigid bacterial cell wall[1]. The primary difference between *Mycoplasma* and other bacteria is that bacteria have a solid cell-wall structure and can grow in the simplest culture media, also bacteria are inhibited by penicillin[2]. The lack of cell wall is used to distinguish these microorganisms from other bacteria and to separate them in a class mollicutes[3]. *M.hominis, Ureaplasma spp*. and *M.genitalium* play a role in prostitis, epididymitis and pyelonephritis[4]**.** *U.urealyticum* can be found in the cervix or vagina of 40-80% of asymptomatic women and *M.hominis* in20-50%[5]. Mycoplasmas are also isolated from the lower urogenital tract of healthy adults, men and women Colonization varies in relation with several parameters including age, race, hormonal status and the lifetime number of sexual partners, and is greater among women, especially during pregnancy[6,7,8] Mycoplasma hominis is the second smallest, self-replicating mycoplasma species that colonizes humans. This facultative pathogenic cell wall-less bacterium is found as a commensal in the urogenital tract of sexually active people, but is also associated with bacterial vaginosis, pelvic inflammatory disease, arthritis and even neonatal meningitis[9]. In men *Ureaplasma spp*.and *M .genitalium* cause non gonococcalurethritis(NGU)[10]

Use of Mycoplasma species-specific DNA probes made it possible to discriminate between different species, this method proved to be rapid and specific[11] According to reviewing of some libraries and internet literatures, this is the first study performe on myco - -plasma of urogenital tract from urine of both males and female in different age in Iraq and middle east ,and it is the first isolation of *M . fermemntas* in Iraq from urine samples.

Aims of this study: Detection of the frequency of mycoplasmal infections by using MDCS method and PCR assay ,explore the role of Mycoplasma in the male and female urinary tract infection and compier the isolation of Myco--plasma with other bacteria.

Material and Methods

Mid stream urine specimens were collected aseptically from 200 patients admitted to Al-Basrah general hospital, Al-Sadr

teaching hospital, Al–*Fayhaa* general hospital and Al- *Mawani* general hospital in center of Basrah city with signs and symptoms suggestive of UTI for the period from 1st November 2012 to April 2013,The age of patients ranged from5-65 years. Another 50 urine samples were collected from patients attending outpatient clinics without signs and symptoms of UTI and considered as control. All specimens were cultured within one hours of sampling. For the isolation of *Mycoplasma*, each urine specimen was directly centrifuge at 2000 rpm for 10 min then loop-full from each sample was transferred to the liquid phase of MDCS was made up of slant solid medium (PPLO) agar, which is covered with 1 ml of (PPLO) broth medium thus, establishing diphasic (sold– liquid) environment at the bottom of the test tube, above which there is a monophasic solid one[12].

Mixed up well and then tited onece or twice to cover the upper portion of slant for a while prior to incubation. All inculated media were incubated aerobically for 2-3 days at 37°C and observed daily for colour change from red to yellow in the liquid phase after 24 hrs. Then isolated colonies appeared after that on slaned solid phase. Blood agar and MacConkey agar other ordinary media were used to know if other bacteria were grown. Bacterial isolated were identified using specific biochemical tests and then confirmed by using molecular diagnosis technique. Dissecting Microscope was used for examination colonial morphology[13]. The colonies of *M. hominis* appear as fried-egg appearance, while the colony of *U. urealyticum* appear granular and dark golden brown color, color due to accumulation of manganese oxide[14,15]. Molecular experiments included the extraction and amplification of *Mycoplasma* and *Ureaplasma* DNA**.** The genus-specific mycoplasma PCR primers table(1) were based on this study described by Hopert et al.[16]. The DNA was extracted according to extraction kit recommended by Geneaid co., Korea and the DNA primers provided by (Bioneer- Korea). PCR assay using universal primers capableof detecting 11 Mycoplasma species were used to target the conserved regin of 16s rRNA[17].

The reaction mixture: Amplification of DNA was carried out in a final volume of 20 µl containing the contents shown in table-2. Detection of amplified products by agarose gel electrophoresis Successful PCR amplification was confirmed by agarose gel electrophoresis as mentioned by Luki N. et al.[18].and photographed under a UV light.

Results and Discussion

The results are based on the study of two hundred urine samples from both males and females *Mycoplasma spp* were isolated from 87 individuals out of 200 patint in this study females affected more than males the commonly affected age group was 15-40 this can attributed to the sexually activity among this group, Culture by MDCS method were used to detect Mycoplasmas pp in genitourinary tract infections and basing on diagnostic characteristics revealed positive results of Mycoplasma spp in 87 cases (43.5%) included; Ureaplasma urealyticum in 73 cases (36.5%) while 13(6.5%) cases were positive for M hominis and Mycoplasma fermentans in one case.(0.5%) as shown in figures 1,2,3. Different species of bacterial isolates other than Mycoplasma were identified in 147 (73.5%) *E. coli* was the first causative agent of infection73 (36.5%) then *Staphylococcus aureus* 40 (20%) *Klepsiella pneumoniae* 25 (12.5*) Pseudomonas aeruginosa 7* (3.5%) *proteus* 2(1%) as shown in table (3)

Detection of mycoplasmal with PCR were identified using universal primers detecting 11species of mycoplasma 73 isolated of *U.urealyticum and 13* isolates of M. hominis were selected for investigation by PCR method. The results of this method indicated that 35/73 (47.9%) isolates of U. urealyticum were positive with PCR method versus 38/73(52%) were negative while among the 13 isolates of M. hominis, only 6/13 (46.1%) were positive with PCR technique versus 7/13 (53.8%) isolates exhibited negative results of this experiment are shown in table 4. Not all these isolates revealed positive results with PCR as in culture method, to show the resuls of this experiment in this paper only seven isolates of U urealyticum were selected. All seven isolates (1-7) of U.urealyticom reseavled positive results by PCR method as shown in figure 4 showing the PCR product result from U urealyticum after run the product in gel.

Table-1
PCR universal primers employed in the detection *Mycoplasma*

Primers Type		Primer sequences	Length	Tm	TA
universal	*F *R	GTG GGG AGC AAA TAG GAT TAG A GGC ATG ATG ATT TGA CGA CAT	22 21	53.8°c 54.4°c	55c° 55°c
universal	F R	GTG GGG AGC AAA CAG GAT TAG A GGC ATG ATG ATT TGA CGT CGT	22 21	56.7c°56.9°c	55°c 55°

Table-2
Contents of the reaction mixture

NO	Contents of reaction mixture	Volume
1	Master Mix	5 µl
2	Premier Forward	2.5 µl
3	Premier Reverse	2.5µl
4	DNA	5 µl
5	Nuclease free water	5 µl
	Total volume	20 µl

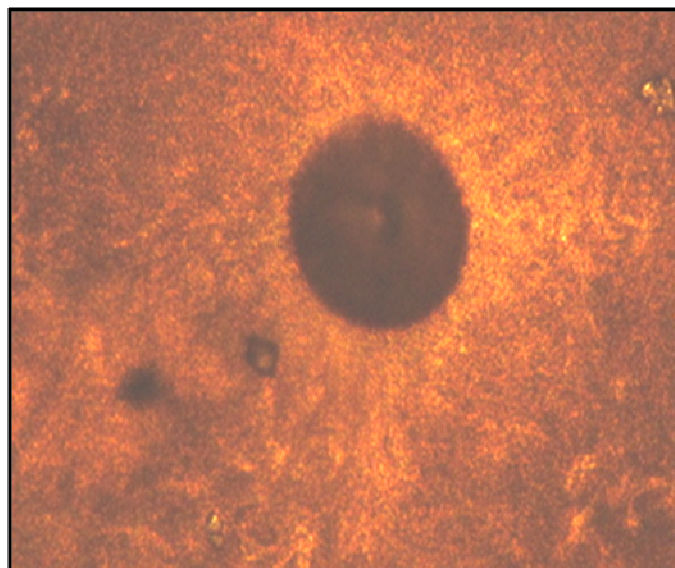

Figure-1
Fried-egg colony of *U . urealyticum*

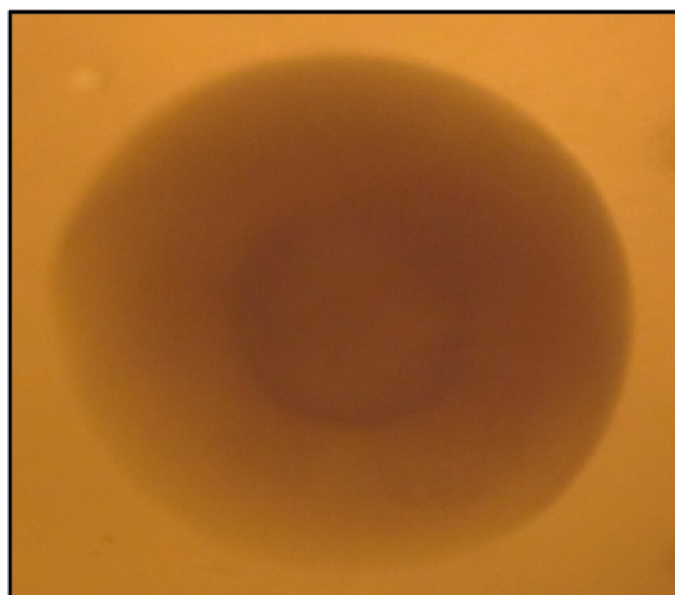

Figure-2
Fried-egg colony of *Mycoplasma hominis*

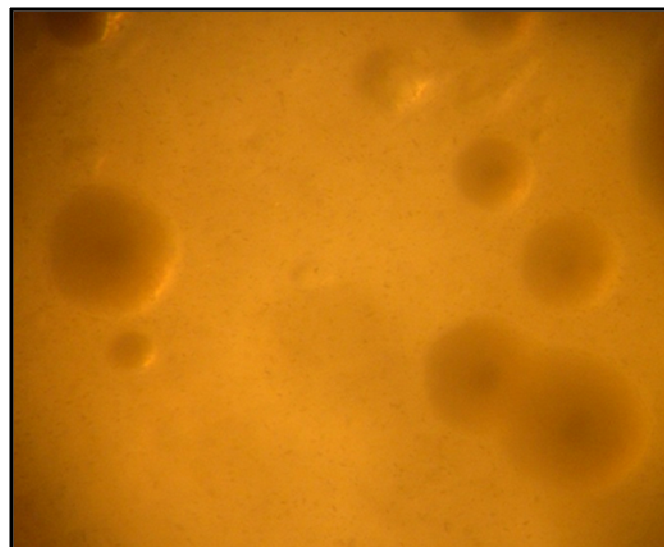

Figure-3
Fried-egg colony of *Mycoplasma fermentas*

Table-3
Mycoplasmas and other causes in patients with UIT

NO. Of isolates (%)	Causative agents
65 (32.5%)	*U. urealyticum*
5 (2.5%)	*M. hominis*
1 (0.5%)	*M. fermentans*
8 (4%)	*U. urealyicum* &M *hominis*
73 (36.5%)	Escherichia coli.
40(20 %)	*Staphylococcus aureus*
25(12.5%)	*Klebsiella pneumoniae*
7(3.5%)	*Pseudomonas aeruginosa*
2(1%)	*Proteus*
44	Negative cultivatation

Table-4
Results of PCR method versus culturing method in detecting *Ureaplasma* and *mycopla*sma

Organisms	Positive cases by culture	Tested cases by PCR	Positive cases by PCR	Negative cases by PC
U. urealyticum	73	73	35	38
M. hominis	13	13	6	7

Electrophoretic analysis of PCR producte for mycoplasma genus from urine sample Lane M10000bp size marker, lane (1-4) *U. urealyticum*, (5,6) M. hominis and (7) M .fermentans: Bacteria other than *Mycoplasma* were isolated in this study they consisted of *Staphylococcus aureus* which was the most frequently isolated bacterial species 40 cases followed by *Klebsiella pneumonia* 19 cases, *E.coli* in 17 cases and *P aeruginosa* in 2 cases as shown in table 5.

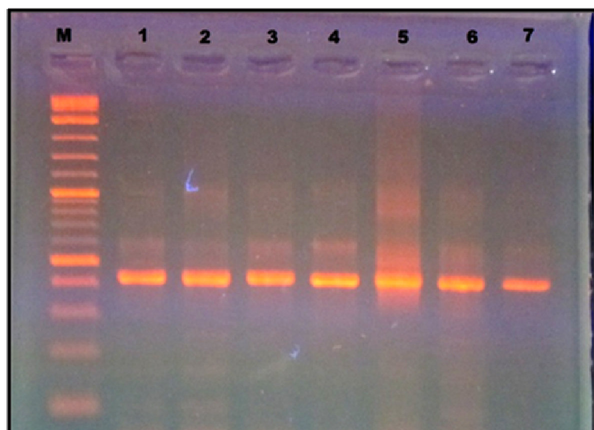

Figure-4
Ethidium bromide stained 2% agarose gel shows the PCR amplification products with universal primer

Table-5
Presence of genital mycoplasma alone or in conjucation with other bacteria

Total Mycoplasma ssp	***Alone***	**Conjucation with**			
		Staphylococcus aureus	*K pneumoniae*	*E. coli*	*P. aeruginosa*
87	9	40	19	17	2

The method of MDCS monophasic-diphasic culture setup was used in this study for the rapid isolation and identifigtion of urogenital mycoplasma from urine specimens. Mycoplasmas were isolated and identified on modified mycoplasmas medium as apathogenic factor for UTI MDCS was used successfully to isolation M. hominis, U. urealyticum and M. fermentas. Simihairi[19] used conventional system represented by an arginine broth and arginine agar culture media to isolate *M. hominis* besides, acommercially prepared medium with a Mycotrim cultivation system wich performed well in isolating *U. urealyticum* and its rather batter than the conventional system but these media were diagnosed with the problem that *U.urealyticum* has sensitivity to the thallium acetate, This problem was resolved by using the modified PPLO medium with a MDCS procedure which is a very efficient differential and selective medium for isolating species of genital Mycoplasma.

On the other hand, AL-Bahli[20] isolated *M. hominis* and *U. urealyticum* from urogenital tract on U9B and A7 agar, but the MDCS appears to be more efficient due to the fact that it consists of two phases broth and agar ,which leads to the formation of three environmental conditions and it proves to be also faster than it . On the other hand Yajko *et al*[21], reported that there were several media for isolation of mycoplasmas and all of these involving horse serum as cholesterol source , which is found to be expensive and difficult to colect .Both of AL-Ghizawi and AL- Mossawi[22] used MDCS medium for the isolation of mycoplasmas and *Ureaplasma urealyticum* which proves to be as efficient compared with the other pervious media. That result comes in an agreement with the present study. AL-Bahli; Simihairi did not isolate *M. fermentans* While, AL-Mossawi isolated this bacteria from urogenital tract in Basrah from high vagina and endocervix swab. In the present study, *M. fermentans* have been isolated from one patient from femal urine sample as the first isolation in Iraq from urine. The overall prevalence of *U. urealyticum* in our group of patients was higher than those of *M. hominis* it is related to sexual activity, hence and sexual active group is apotential carrier. Asmilar result was obtained by Zdrodwskastefanow et al[23].

This study most of the patients presenting with lower urinary tract symptoms had a positive testing for bacteria illustrating the importance of ruling out these specific infections, which are not detected by routine microbiological tests and need to be treated with specific antimicrobiotics[24] that confirm with Latthe ,et al[25]. PCR has been used to detect a number of Mycoplasma species. However, with over 102 mycoplasmas currently recognized it is not feasible to develop PCR tests for each species and there is need for a single generic test that can both detect and differentiate mycoplasmas. Selection of a variety of target sequences, starting with highly conserved regions of the genes, allowed design of primers of wide specificity ("universal primers") for detection of Mycoplasma infections in anatomic sites where at least 2 or 3species are frequently found. The use of a single Mollicutes universal primer set in cases of life-threatening infections has the advantage of allowing a rapid positive or negative report to clinicians, and in turn to establish as soon as possible the appropriate treatment[26] Indicating speci¢city of the PCR assay it was not observed any PCR result that was positive for Mycoplasma genome but negative for Mycoplasma cutures. Use of Mycoplasma species-specific DNA probes made it possible to discriminate between different species, this method proved to be rapid and specific [27]

Conclusion

The modified PPLO medium with its Monophasic- Diphasic Culture Setup (MDCS) pattern is a very efficient differential and selective medium for isolation species of genital Mycoplasma including :M. *hominis*, *M. fermentans*, *U. urealyticum*.

Both *U. urealyticum* and *Mycoplasma* are frequently infect genitourinary tract of males and females .In this study the incidence rate of genitourinary infaction due to *U.urealyticum* was conciderably higher as compared *to M. hominis*. Detected by culture isolation which should be eventually confirmed further by PCR assay.

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