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The association between vaginal colonization by mycoplasma and adverse pregnancy outcomes

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ABSTRACT

Preterm birth & and low birth weight are a major health care problem and are significant contributors to perinatal morbidity, mortality, and long-term disability and ascending genital tract infection is a major cause of preterm labor. This study was aimed to examine the association between vaginal colonisation by genital Mycoplasma bacteria species of (Mycoplasma hominis and Ureaplasma urealyticum) of a pregnant patient with subsequent development of adverse pregnancy outcomes such as preterm labor (PTL), preterm premature rupture of membrane (PPROM) and chorioamnionitis. A prospective cohort study included 75 pregnant patients with gestational age of (24-34) weeks attending the Obstetrics and Gynecology Department in Al-Diwaniyah Maternity and Pediatric Teaching Hospital, Iraq, from (February 2018- December 2018) who will exhibit obstetrical symptoms and signs of abdominal pain, uterine contractions, or cervical length shortening on transvaginal ultrasound (<25 mm), vaginal samples collected from those pregnant patients and all samples were tested for the presence Mycoplasma hominis and Ureaplasma urealyticm bacteria by using molecular diagnosis method of polymerase chain reaction (PCR) and follow up of the patients for the development of adverse pregnancy outcomes as preterm labor, premature rupture of membrane and chorioamnionitis. The results of the current study revealed that the rate of Mycoplasma hominis infection was 18.2 %, whereas that of Ureaplasma was 7.6 %. It has been concluded there is an association between genital Mycoplasma in initiating adverse pregnancy outcome.

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INTRODUCTION

Preterm birth (PTB) and low birth weight are major health care problems and are significant contributors to perinatal morbidity, mortality, and long-term disability. Preterm birth is defined as birth at ≥ 24 weeks and before a 37completed week with regular contractions of the *uterus* resulting in changes in the *cervix* that include effacement (the cervix thins out) and dilation (the cervix opens so that the *fetus* can enter the birth canal) (Romera *et al.,* 2014). There are sub-categories of preterm birth, based on gestational age:

- extremely preterm (less than 28 weeks)
- very preterm (28 to 32 weeks)
- moderate to late preterm (32 to 37 weeks)

Over the past few decades, the perinatal outcomes of preterm neonates have improved markedly through research and advances in neonatal care, whereas rates of spontaneous PTB have essentially remained static (Rubens *et al.,* 2014). Preterm birth is the major cause of death and disability in children up to 5years of age in the developed world, and the single leading cause of global perinatal mortality and morbidity, approximately 15

million babies are born preterm each year worldwide, and a million of those children die (Harrison *et al.,* 2016).The preterm infant is also at significantly greater risk of serious perinatal complications, while many children born preterm had a normal and healthy life, a significant proportion experience lifelong disability and health issues. Chorioamnionitis is a common cause of preterm birth. Clinical chorioamnionitis, characterized by maternal fever, leukocytosis, tachycardia, uterine tenderness, and preterm rupture of membranes, is less common than subclinical/histologic chorioamnionitis, which is asymptomatic and defined by inflammation of the chorion, amnion, and placenta. Chorioamnionitis is often associated with a fetal inflammatory response syndrome (FIRS) which is defined by increased systemic inflammatory cytokine concentrations, funisitis, and fetal vasculitis which leads to poor cardiorespiratory, neurological, and renal outcomes (Shah *et al.,* 2016). The mechanism of preterm labor (PTL) is multifactorial, and in developed countries nearly 12% of all deliveries were preterm. About 30% of preterm deliveries are iatrogenic for both maternal and fetal conditions as (pre-eclampsia, placental abruption or placenta previa, intrauterine growth restriction &fetal distress) with the remainders being spontaneous, either with an intact membrane or following preterm pre labor rupture of the membranes (PPROM) (Romero *et al.,* 2006). The causes of spontaneous PTL in most cases are due to genitourinary tract infection, placental vascular abnormalities as placenta Previa or placental abruption, uterine overdistension, and uterine anomalies. Several risk factors for PTL have been identified, including poor socioeconomic status, smoking, low education levels, maternal age <20years and >35years, heavy maternal work, cervical incompetence, previous obstetrical history of abortion and preterm labor (AL-Dabbagh and AL-Taee, 2006). An estimated 25% - 40% of preterm births are attributed to intrauterine infection, and ascending genital tract infection is a major cause of preterm labor (Kim *et al.,* 2015; Cram *et al.,* 2002). Genital mycoplasmas are very prevalent causes of various female genital tract infections such as vaginitis, cervicitis, and pelvic inflammatory disease (PID), infertility, pyelonephritis, and septicaemia. In addition to major maternal and perinatal morbidities such as PTL, spontaneous abortion, fetal respiratory distress syndrome and fetal inflammatory response syndrome (Schlicht *et al.,* 2004). Mycoplasma hominis and Ureaplasma urea lyticum are members of the Mycoplasmataceae family. These bacteria characterized by their lack of a cell wall and have small genomes and are often dependent on their host with limited biosynthetic abilities, and through their cell surface appendages these organisms attached to genital tract epithelium and due to high degree of colonization in endocervical tissues, they induce various complications in pregnant women and in newborn as well (Taylor_Robinson, 2007). Colonization varies in relation to several parameters including age, race, hormonal status and number of sexual partners, and is greater among women, especially during pregnancy. The potential pathogenic role of these bacteria during pregnancy was recently reviewed, as these organisms may be associated with adverse pregnancy outcomes such as preterm labor, preterm premature rupture of membranes (PPROM), chorioamnionitis, abortion and low birth weight (Lawton *et al.,* 2008). Because genital mycoplasmas lack a cell wall, they are resistant to antimicrobial agents that are active against this structure. Therefore, penicillins, cephalosporins, and vancomycin are ineffective in the treatment of conditions caused by these microorganisms. Historically, the most common antimicrobials active against mycoplasmas are included in the three major drug classes: tetracyclines, macrolides, and quinolones have been the major antibiotics used in the treatment of urogenital infections caused by mycoplasma. However, their therapeutic efficacy may be unpredictable due to increasing resistance (Krausse and Schubert, 2010). During pregnancy, the list of antibiotics that are not contraindicated but effective against urogenital mycoplasmas is much shorter. Macrolides (erythromycin and azithromycin) are allowed during pregnancy. Erythromycin, the antibiotic most commonly used for treating pregnant women, has shown only moderate activity because of increasing resistance. Azithromycin antibiotic inhibits bacterial protein synthesis, can be used during pregnancy (Redelinghuys *et al.,* 2014). In order to elucidate the potential role of genital mycoplasma infections in pregnant and new borne, it is important to utilize a fast and efficient bacterial detection system. In the past, bacterial culture was considered to be the standard gold method for mycoplasma detection. Culture is however very tedious, of high cost as it needs a specialized media for growth and time consuming taking 2 to5 days for Ureaplasma urealyticum and Mycoplasma hominis (Redelinghuys *et al.,* 2014; Obata *et al.,* 2002).Various DNA amplification techniques have proven to be a suitable alternative to culture for detection of genital mycoplasmas in clinical samples which includes polymerase chain reaction test (PCR), DNA probe, and enzyme immunoassays Obata *et al.,* 2002). Real-time PCR (also known as quantitative or qPCR) allows accurate quantification of starting amount of DNA and RNA targets, then fluorescence is measured during each cycle, and the amount of fluorescence is proportional to the number of PCR products using either fluorescent dyes that bind to double-stranded DNA (like the DNA binding dye

SYBER Green 1) or fluorescently labelled sequence-specific probes oligonucleotide (Cunningham *et al.,* 2013, Waites *et al.,* 2012). So, this study was designed to examine the association between vaginal colonization of pregnant patients with genital mycoplasma bacteria (Mycoplasma hominis and Ureaplasma urealyticum) with subsequent development of adverse pregnancy complications as PTL, PPROM, and chorioamnionitis by using molecular PCR say.

PATIENTS AND METHODS

This study was a prospective cohort study that involves 75 pregnant women attending the Obstetric Department at Al-Diwaniyah Maternity and Children Teaching Hospital, Iraq, from the period of (February-2018 to December-2018), with gestational age between 24-34weeks, who exhibited obstetric symptoms and signs of abdominal pain, uterine contractions, and cervical length shortening on transvaginal ultrasound<25mm, vaginal samples were collected, and all samples were tested for the presence of *M.hominis* and *U.urealyticum* by PCR test, and follow up of the patients for the development of any adverse pregnancy outcomes, like preterm labor, P PROM and chorioamnionitis. The study was approved by the Iraqi Ethical Committee of the University and the Hospital, and informed consent was taken from all patients prior to participation in the study.

Exclusion criteria

This exclusionary regimen facility the ability to study the presence of genital infection by M.hominis and U.urealyticum bacteria as a sole risk factor in adverse pregnancy complications, there for women with any of the following risk factor for preterm birth were excluded from the study: Patients age <18years>45years, cervical dilatation ≥3cm at time of enrollment, ruptured membranes, placenta previa, placental abruption, multiple gestations, polyhydramnios, oligohydramnios, fetal congenital malformation, intrauterine fetal death, intrauterine growth restriction, maternal medical conditions as diabetes mellitus, gestational diabetes, chronic hypertension, maternal cardiac disease, preeclampsia and pregnancy-induced hypertension, Rh-isoimmunization, uterine fibroid and anomalies, abdominal trauma, in vitro fertilization, maternal renal disease and urinary tract infection, bacterial vaginosis infection, Trichomonas vaginalis infection, and group B-streptococcal infection at the time of enrollment. The patients were admitted to the obstetrical department and initially examined, and the gestational age was determined by a combination of the last menstrual period and ultrasonographic evaluation. Other demographic data regarding patients age, parity, body mass index (BMI) kg/m^2, and any previous obstetrical history related to preterm labor were recorded. Investigations did for all patients including Complete blood picture (CBP), random blood sugar (RBS), mid-stream urine examination, renal function test (RFT), liver function test (LFT), high vaginal swab (HVS) done for culture and sensitivity to exclude other pathogenic infection.

Sample preparation and PCR test

A sterile speculum examination was performed to collect vaginal fluid from the upper one-third of the vaginal sidewalls and posterior vaginal fornix using commercial tubes kit (Universal transport media-UK) of 1-2 ml of vaginal fluid into sterile sample cylinder and labelled patient name, then coated in the ice bag until transferred to the laboratory refrigerator (LG-Korea) stored in -20 C° then prepared for DNA extraction. Then the frozen specimens were thawed by water path 37C° (Siemens – Germany) on the day the assays were performed, and extraction of DNA from clinical samples was performed by using DNA-extraction kit (Promega – USA). The extracted DNA was examined for M. hominis and U. urealyticum by using Mycoplasma and Ureaplasma species-specific PCR Briefly, 1ml of the sample was centrifuged (Hitsch-Germany) at 12000 ×cycle for 10 min. The pellet washed in PBS (phosphate – buffer saline) to maintain constant pH and resuspended in 50µ l of distilled water. After boiling 80C° for 10 min, an aliquot of 7µl was used.

PCR assay

PCR reactions were performed with an automated thermal cycler (Rotor-Gene Q) real-time PCR (QIAGEN - Germany).

The result was received within 1 hour and positive amplification passing the threshold line before 35 cycles considered a positive result, and this result is double checked by melting curve analysis to detect the accuracy of the amplification. Tocolytics and dexamethasone were given as appropriate, and patients whose samples were positive for one or both bacteria were treated with azithromycin table (Roxithromycin –ROX PHARMA –Netherland) (500mg x1) per day for 5 days and follow up of the patients for the development of any adverse pregnancy complications as PLT, PPROM, or chorioamnionitis. Seventy-five patients were enrolled in the study, but only 66 patients were followed as 9 patients were lost to follow up.

Statistical analysis

Data were summarized, analyzed and presented using Statistical Package for Social Sciences (SPSS, version 23) and Microsoft Office Excel 2010.

Table 1: Nucleotide sequences of the primers used

Primers and target DNA	Nucleotide sequences (5'-3')	Amplicon length & Reference
Urease gene of U. urealyticum		
U4 forwarded primer	ACGACGT CCATAAGCAACT	429bp (13)
U5 reverse primer	CAATCTGCTCGTGAAGTATTAC	
16SrRNA gene of M. hominis	CAA TGG CTA ATG CCG GAT ACG C	344bp (14)
RNAH1 forwarded primer	CAA TGG CTA ATG CCG GAT ACG C	344bp (14)
RNAH2 reverse primer	GGT ACC GTC AGT CTG CAA T	

Table 2: PCR amplification reaction setup

Component	reaction mixture	final concentration
Luna Universal qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	0.5 µl	0.25 µM
Reverse primer (10 µM)	0.5 µl	0.25 µM
Template DNA	2µl	< 100 ng
Nuclease-free Water	20 µl	

Table 3: Thermal cycle steps of PCR amplification

Cycle steps	Temperature	Time	Cycle number
Initial Denaturation	95°C	60 seconds	1
Denaturation	95°C	15 seconds	40-45
Extension	60°C	60 seconds	
Melt Curve	60-95°C	45 second	1

Qualitative variables were expressed as number and percentage whereas quantitative variables were expressed as mean and standard deviation in case of normally distributed data; median and inter-quartile ranges were used in case of non-normally distributed data. Comparison of numeric variables between any two groups was conducted using independent samples t-test in case of normally distributed data, and Mann Whitney U test in case of non-normally distributed data. Chi-square test was used to study the association between categorical variables; in the case when the Chi-square test was not valid, Yates correction for continuity was used instead. Estimation of risk was performed according to the odds ratio calculation. The level of significance was set at $P \leq 0.05$.

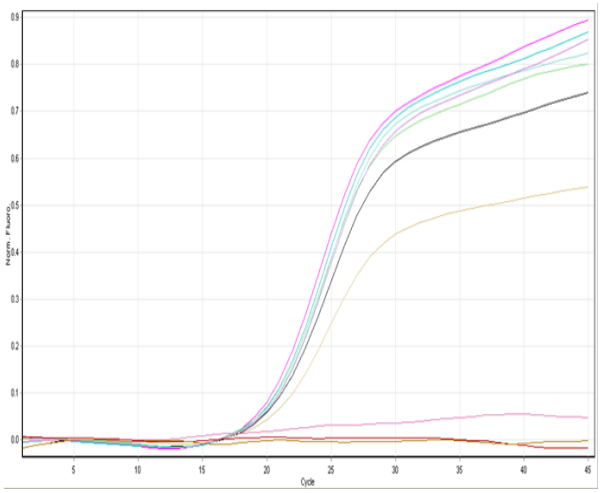

Figure 1: Amplification curve of Mycoplasma hominis infection

RESULTS

The age of the patients in our study was in the range of 20 to 38 years with a mean of 27.97 ±4.57 years. The body mass index of the patients ranged from 18 - 25 kg / m^2 and the mean BMI was 21.05 ± 1.96 kg / m^2. Gestational age varied from 25 to 34 weeks with a mean of 30.09 ± 2.31 weeks. Overall, parity ranged from 0 to 4, and the median parity was 2. History of previous single pre-term labor was seen in 24.2 % of women, and a history of 2 previous preterm labor was seen in 3.0 % of women, as shown in (table 4). Real-time PCR amplification results passing the threshold line before 35 cycles considered as a positive result of infection and this result is double checked by melting curve analysis to detect the accuracy of the amplification as shown in figure 1, 2, 3, 4.

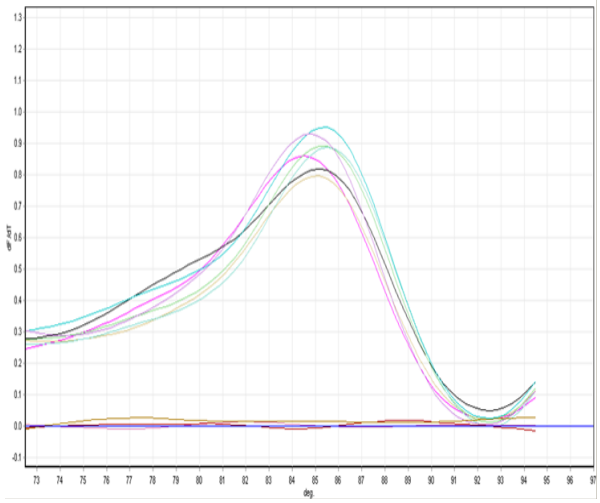

Figure 2: Melting curve analysis of Mycoplasma hominis infection

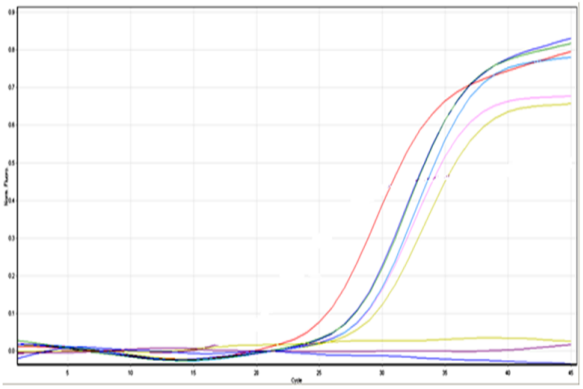

Figure 3: Amplification curve of Ureaplasma urealyticum infection

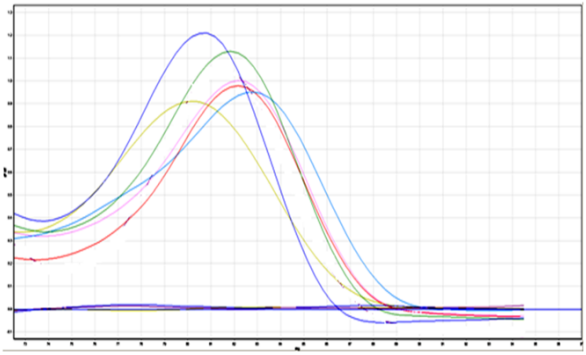

Figure 4: Melting curve analysis of Ureaplasma urealyticum infection

Table 4: General characteristics of women enrolled in the present study

Characteristic	Value
N	66
Age (years)	
Range	20 - 38
Mean ±SD	27.97 ±4.57
BMI (kg / m2)	
Range	18 - 25
Mean ±SD	21.05 ± 1.96
Gestational age (weeks)	
Range	25 - 34
Mean ±SD	30.09 ± 2.31
Parity	
Range	0 - 4
Median (IQR)	2 (2)
Para (0)	14 (21.2 %)
Para (1-3)	48 (72.7 %)
Para (> 3)	4 (6.1 %)
History of preterm labor	
0	48 (72.8 %)
1	16 (24.2 %)
2	2 (3.0 %)

Table 5: Rate of infection with *Mycoplasma* and *Ureaplasma*

Infection	*n*	%
Mycoplasma	12	18.2
Ureaplasma	5	7.6
Negative	49	74.2

Table 6: Rates and types of complications

Complication	*n*	%
PTL	15	22.7
PPROM	6	9.1
Chorioamnionitis	2	3.0
No complications	43	65.2

The age of the patients in our study was in the range of 20 to 38 years with a mean of 27.97 ±4.57 years. The body mass index of the patients ranged from 18 - 25 kg / m^2 and the mean BMI was 21.05 ± 1.96 kg / m^2. Gestational age varied from 25 to 34 weeks with a mean of 30.09 ± 2.31 weeks. Overall, parity ranged from 0 to 4, and the median parity was 2. History of previous single pre-term labor was seen in 24.2 % of women, and a history of 2 previous preterm labor was seen in 3.0 % of women, as shown in (table 4). The rate of Mycoplasma infection was 18.2 %, whereas that of Ureaplasma was 7.6 %, as shown in (table 5). Complication encountered during the period of follow up included preterm labor in 15 women (22.7 %), premature rupture of the membrane in 6 women (9.1 %) and chorioamnionitis in 2 women (3.0 %), as shown in table 6. Complications were seen significantly in younger women, 26.39±4.80 years versus 28.81 ±4.26 years (P = 0.039), as shown in table 7. There was a significant association between genital Mycoplasma, and the development of pregnancy complications in 64.7% with (p=0.003) and OR 5.65 with 95% CI of (1.72 - 18.56) and more complications rate was associated with Ureaplasma infection (P =0.046) (table 8).

DISCUSSION

In this study, a real-time PCR assay was used to detect two pathogenic species of genital mycoplasma as detection of Mycoplasma species is clinically relevant in light of the increased capability of the bacteria to adhere and colonize the endocervical lining and thereby inducing adverse clinical symptoms in women, fetus as well as in newborns. Our study showed that maternal age, GA, parity, BMI and the history PTL had on the significant association for preterm labor in pregnant women tested for genital mycoplasma colonization with Mycoplasma hominis and Ureaplasma ureaiyticum. (p≤0.05). While a study by M. Vouga *et al.,* (2014), showed that higher incidence of Mycoplasma hominis and Ureaplasma urealyticum in young maternal age 29.7±6.0 years and more with BMI>30 kg/m. The controversy in these results may be due to variation in maternal behavioural and physiological traits and to the host-specific characteristics that affect preterm birth via the medication of the vaginal bacterial community as by using douching before pregnancy. In this study, 34.8% had developed complications and the rate of genital mycoplasma

Table 7: Association between complications and other characteristics of the study group

Characteristic	Complications		*P*
	Positive	Negative	
Age (mean ±SD), years	26.39 ±4.80	28.81 ±4.26	0.039S
BMI (mean ±SD), kg / m^2	20.96 ±2.23	21.09 ±1.84	0.790NS
Gestational age (mean ±SD), weeks	30.39 ±2.04	29.93 ±2.44	0.443NS

SD: standard deviation; S: significant at P ≤ 0.05; NS: not significant at P ≤ 0.05

Table 8: Association between complications and infection

Characteristic	Total *n* = 66	Complication		*P*	OR	95 % CI
		Positive *n* = 23	Negative *n* = 43			
Infection	17	11 (64.7 %)	6 (35.3 %)	0.003	5.65	1.72 - 18.56
Mycoplasma	12	7 (58.3 %)	5 (41.7 %)	0.092	3.33	0.92 - 12.05
Ureaplasma	5	4 (80.0 %)	1 (20.0 %)	0.046	8.84	0.93 - 84.51

n: number of cases: OR: odds ratio: CI: confidence interval

infections was 25.8% as detect by PCR of which 18.2% had Mycoplasma hominis genital infection whereas that of the Ureaplasma urealyticum was 7.6%, and this was relatively similar to the finding of Stellrecht *et al.,* study (2004), which proved that PCR is a suitable method for genital mycoplasma detection, as the rates of Mycoplasma and Ureaplasma detection were 20% and 44.3% respectively (Stellrecht *et al.,* 2004). Similarly, N. Amirmozafari, *et al.,* (2009) showed that of the 210 women with vaginal infection, genital swab test was positive in 120 samples as (57.1%) showed positive reaction in PCR of which 32.3%were positive for Ureaplasma urealyticum, 13.3% for Mycoplasma hominis and 11.9% had mixed infections (Amiymozafari *et al.,* 2009). In this study, the complications encountered during the period of follow up included preterm labor in 15 women (22.7%), PPROM in 6 women (9.1%), and chorioamnionitis in 2 women (3.0%) and more complication rate encountered with Mycoplasma hominis infection. The 15 women who had PTL, 6 patients (40%) had a genital infection as 3 women (20%) had Mycoplasma hominis, and 3 women (20%) had Ureaplasma urea lyticum infection. Of the 6 patients who had PPROM, 2 patients (33.3%) had Mycoplasma infection and 1 patient 16.7% had Ureaplasma. The two patients who had developed chorioamnionitis complication, both were positive for Mycoplasma infection only. Additionally, the total infection rate was 17 women, those who developed complications with infection were 11 (64.7%) which was significant (p<or=0.05). Mycoplasma infection rate detected in our study was 12 patients, of which 7 patients developed complications (58.3%) which was relatively high, but it does not reach a statistical significance (p>0.05). While the rate of Ureaplasma infection was 5 patients, of which 4 patients (80%) had developed complications which were significant (p<or=0.05). These results were similar to study by Lee SE, *et al.,* (2009), that was done on nearly 2000 women, who found a preterm birth rate was 4.9%, and 53.6% of those who delivered prematurely showed Ureaplasma urealyticum colonization (Lee *et al.,* 2009). Kasper *et al.,* (2010), showed that of the 225women with PPROM, 68% had cervical colonization by Ureaplasma compared to 17% among control patients, and 28% of PPROM patient was colonized by Mycoplasma hominis compared to 15%among control patients. A study by Waites *et al.,* (2005), showed that of 977 pregnancies, 14% was associated with preterm birth, and Ureaplasma colonization was found in 88%of them while Mycoplasma hominis was present in only 3%. The molecular methods have revolutionized our understanding of these microorganisms because When culture and molecular detection are used simultaneously, methods such as PCR seem to offer great sensitivity as noted by Oh *et al.,* (2010) and Kacerovsky *et al.,* (2009), as cultivation missed most genital mycoplasma present in genital tract tissues in women with placental insufficiency and PPROM, as 91% of women with PCR evidence of Ureaplasma and Mycoplasma had negative cultures for the organisms. Smorgick *et al.,* (2007) showed that in case of premature labor at 27 weeks of gestation, which is associated with Ureaplasma urealyticum colonization treatment with azithromycin or clindamycin prolonged the pregnancy until 33 weeks when spontaneous labor occurred. Similarly, Grigsby *et al.,* (2012) observed that treatment with azithromycin or clindamycin prolonged the pregnancy for 22 days.

CONCLUSION

There is an association between genital Mycoplasma in initiating adverse pregnancy outcome and the use of PCR assay system is simple, fast and highly specific for genital mycoplasma detection and can be a suitable alternative to other Mycoplasma identification methods.

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