

Original Article**Is there an association between recurrent spontaneous abortion and mycoplasma infection?**Jie Yu¹#, Shanshan Yu²#, Liye Zhu¹, Xuan Sun³, Boqi Lu¹, Jian Li⁴, Yuecheng Hu⁵, Peijun Li⁴¹ Department of Obstetrics and Gynecology, Haidian Maternal and Child Health Care Hospital, Beijing, China² Department of Pharmacy, Fifth Medical Center of PLA General Hospital, Beijing, China³ Department of Laboratory, Haidian Maternal and Child Health Care Hospital, Beijing, China⁴ Intensive Care Unit, Tianjin Chest Hospital, Tianjin, China⁵ Department of Cardiology, Tianjin Chest Hospital, Tianjin, China

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Abstract

Introduction: Recurrent spontaneous abortion (RSA) is an important reproductive health issue with a serious adverse effect on patients and their families worldwide. The present study evaluated the association between mycoplasma infections and RSA in pregnant patients.

Methodology: This case-control study included 107 patients with RSA (study group) and 89 normal pregnant women who had planned abortions (control group) between March 2019 and February 2021. Cervical swabs were assessed for the presence of *Mycoplasma hominis* and *Ureaplasma urealyticum* by Microtiter Plate Hybridization assay.

Results: A total of 52 (48.6%) patients from the study group and 13 (14.6%) patients from control group were positive for mycoplasmas. The presence of *M. hominis* (29.9% vs. 9%; $p = 0.024$), *U. urealyticum* (18.7% vs. 5.6%; $p = 0.015$) and the co-infection of *M. hominis/U. urealyticum* (14% vs. 1%; $p = 0.032$) were significantly higher in the study group. Multivariate analysis revealed that pelvic pain (Odds Ratio [OR] = 3.42; 95% CI = 0.40-3.65; $p = 0.015$), dysuria (OR = 4.12; 95% CI = 1.59-8.23; $p = 0.021$), and urinary tract infection (OR = 3.97; 95% CI = 1.52-4.17; $p = 0.032$) were independent predictors of RSA.

Conclusions: The high prevalence of *M. hominis/U. urealyticum* in this study reveals a significant association with RSA. Pelvic pain and Mycoplasma infections are independent predictors of RSA.

Key words: Abortion; mycoplasma; ureaplasma.

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Introduction

Recurrent spontaneous abortion (RSA) is an important reproductive health issue with a serious adverse effect on patients and their families, worldwide. It occurs in about 5-20% of pregnant women and among those about 10-15% of patients experience it even after appropriate treatments [1]. About 14% of pregnancy-related deaths were due to RSA, which accounts for 40,000 pregnancy-related deaths annually [2]. RSA is defined as ‘two or more successive abortions before 20 weeks from the last menstruation [3]. Certain abnormalities include parental chromosomal abnormalities, immunologic abnormalities, uterine anatomic abnormalities, endocrine disorders, acquired thrombophilias, heredity, uncontrolled diabetes mellitus, untreated hypothyroidism, antiphospholipid antibody, environmental factors and infections are reported as the possible cause of RSA [4,5].

Lactobacillus sp. constitutes about 90-95% of the vaginal commensal bacteria, while *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma hominis* were frequently associated with RSA. When abortion occurs due to infection of placental tissue it is termed as septic abortion. It occurs commonly due to vaginal bacteria and the prevalence ranges from 5-20% in several developing countries [2]. The recurrent occurrence of these vaginal bacteria activates the endometrial immunocytes leading to an intense immune response producing excessive Th1 cytokines and having a negative effect during the early stage of pregnancy. The Th2 cytokine is associated with a successful pregnancy, while the presence of excessive Th1 cytokine will have a detrimental effect on pregnancy. The undesirable cytokine and bacterial toxins may lead to placental insufficiency, uterine contraction, amnionitis, life-threatening malformation,

or even death. These may cause abortion or preterm labour in the first or third trimester respectively [6].

Mycoplasma sp. is one of the most common bacteria isolated from cord blood, amniotic fluid, and cerebrospinal fluid of prematurely born infants who develop bronchopulmonary dysplasia and developmental disabilities [7]. Mycoplasmas are the smallest organisms devoid of the cell wall with autonomous growth. Compared to most bacteria, they have a smaller genome and require complex media to grow. *M. hominis* (MH) and *U. urealyticum* (UU) infections are common causes of RSA [6]. These organisms are associated with other adverse pregnancy outcomes including postpartum endometritis, stillbirth, miscarriage, infertility and chorioamnionitis [8]. A study reported that MH and UU colonization in RSA patients was higher when compared to women with successful outcomes of their pregnancies. The study also reported that the infection rate of MH and UU increases in patients who had a higher number of spontaneous abortions. The MH and UU infections were reported to arrest embryonic development [9]. *M. hominis* was also reported as normal genital flora, raising controversies regarding their role in adverse pregnancy outcomes [8]. In addition, due to its fastidious and asymptomatic nature *Mycoplasma* is often missed during the diagnosis. This study evaluated the association between mycoplasma infections and RSA.

Methodology

Patients

A case-control study was conducted between March 2019 and February 2021 in Tianjin Chest Hospital, Tianjin, China. Pregnant women who were admitted to hospital wards and/or the intensive care units were included in the study. One hundred and seven patients with a mean age of 28 ± 5.6 years who were diagnosed with RSA were included in the study group. Eighty-nine normal pregnant women with a mean age of 26.3 ± 4.3 years who had planned abortions in the same period were included as a control (control group). In the study group, spontaneous abortion occurred between 6 and 13 gestational weeks (mean 9.8 ± 2.7 weeks) and in the control group planned abortion took place between 5 and 12 gestational weeks (mean 8.7 ± 3.4 weeks). Of the 107 patients in the study group, 79 patients had 2 abortions and 10 patients had 3 abortions. For the study group patients with more than two spontaneous abortions were included in the study. Information regarding the gestational period of the previous abortion was obtained from the medical records

wherever available, or else from patient self-report. Patients who were on antibiotics within 2 weeks of sample collection were excluded from the analysis. Demographic data including age, marital status, menstrual characteristics, pathological history and sexual history were collected. Informed consent from all patients was obtained before the sample collection. The institutional ethics committee approved the study (HEC-012-2018).

Study design and patient population

A case-control study was conducted between March 2017 and February 2021 in Haidian Maternal And Child Health Care Hospital, Beijing, China. Pregnant women who were admitted to hospital wards and/or the intensive care units were included in the study.

Sample collection

Cervical swabs were collected from all the patients with and without RSA after the spontaneous abortion in the study group and during the abortion procedure in the control group. All samples were transported to the laboratory in 4 ml of transport media. The samples were then homogenized and aliquoted to 1 ml each and stored at -20°C until processed.

DNA Extraction

The stored samples were extracted for DNA using QIAamp DNA Blood and Tissue Extraction Kit (Qiagen, Milan, Italy) as per the manufacturer's instructions. NanoDrop 2000 (Thermo Fischer Scientific, USA) was used to quantify the extracted DNA. The extracted DNA was stored at -80°C until further processed by PCR.

Polymerase chain reaction

M. hominis and *U. urealyticum* were detected using species-specific microtiter plate hybridization assay as described by Yoshida *et al.* [10]. The PCR was performed to amplify the 16s rRNA gene of *M. hominis* and *U. urealyticum* using the following primers: forward primer, My-ins ($5'$ -GTAATACATAGGTGCAAGCGTTATC- $3'$), and two biotinylated reverse primers, MGSO-2-Bi ($5'$ -biotin-CACCACATCTGTCACTCTGTTAACCTC- $3'$) and UGSO-Bi ($5'$ -biotin-CACCACCTGTCATATTGTTAACCTC- $3'$). PCR was performed using a 25 μL master mix containing 2.5 μL of template DNA, 0.2 μM (2 μL) of each primer, and 12.5 μL of TEMPase Hot Start Master Mix (Amplicon, Denmark) and 6 μL of DNase/RNase free water. The following PCR cycling conditions were used: initial

denaturation at 95 °C for 10 minutes followed by 50 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute and a final extension step at 72 °C for 7 minutes. After PCR, 3 µL of the PCR product with an expected amplicon size of 520 bp were resolved in 2% agarose gel electrophoresis and visualized under UV transilluminator to compare the results of hybridization. The remaining PCR product was stored at -20 °C until used for PCR Microtiter Plate Hybridization assay.

Microtiter plate hybridization

The amplified PCR products were hybridized using species-specific human mycoplasmas and ureaplasma probes. Mhom-P10-Am (5'-GACACTAGCAAACTAGAGTTAG-3') specific for *M. hominis* and Uure-P4-Am (5'-GGCTCGAACGAGTCGGTGT-3') specific for *U. urealyticum* were hybridized on microtiter plates as described by Yoshida *et al.* [10]. After the hybridization, the reaction mixture was measured for its OD value at 450 nm using xMark™ Microplate Absorbance Spectrophotometer (BioRad, Singapore). For negative control, the OD value was determined to be 0.280 as the cut-off value after measuring 15 times. A sample was considered positive if the OD value was ≥ 0.280, and negative if the OD value < 0.280.

Statistical analysis

Data with continuous values were represented as mean, median and ranges, categorical values were represented as numbers and percentages. Mann-Whitney U test, Fisher's exact probability test, and

multivariate logistic regression analysis (SPSS; SPSS Inc., Chicago, IL) were performed. A P value of <0.05 was considered to be statistically significant.

Results

Of the included patients, majority of the patients were in the age group of 21-30 years in both study (76, 71.0%) and the control (67, 75.3%) groups. The age of sexual onset for majority of patients was >20 years in the study (52, 48.6%) and control (56, 62.9%) groups. The presence of clinical conditions/symptoms (study vs. control groups) including vaginal discharge (66.4% vs. 25.8%; $p = 0.019$), vaginal itching (83.2% vs. 34.8%; $p = 0.016$), pelvic pain (38.3% vs. 10.1%; $p = 0.034$), dysuria (27.1% vs. 7.9%; $p = 0.043$), urinary tract infection (48.6% vs. 15.7%; $p = 0.012$) and irregular menstrual cycle (32.7% vs. 13.5%; $p = 0.017$) were significantly higher in the study group. There was no significant ($p > 0.05$) difference in other patient characteristics between both the groups (Table 1).

Of the 107 patients in the study group, 52 (48.6%) patients were positive for *Mycoplasma* sp., while in the control group 13 (14.6%) patients were positive by PCR microtiter plate hybridization assay. The presence of *M. hominis* (29.9% vs. 9%; $p = 0.024$), *U. urealyticum* (18.7% vs. 5.6%; $p = 0.015$) and the co-infection of *M. hominis/U. urealyticum* (14% vs. 1%; $p = 0.032$) was significantly higher in the study group (Table 2).

Among the patients with various clinical conditions, the presence of any *Mycoplasma* sp. was significantly higher in patients with pelvic pain (41.5% vs. 22.2%; $p = 0.027$) and dysuria (55.2% vs. 14.3%; $p = 0.012$) (Table 3). Multivariate analysis revealed that

Table 1. Patient characteristics and demographic details.

Patient characteristics	Study group (n = 107)	Control group (n = 89)	p-value
Mean age (± SD) years	28 ± 5.6	26.3 ± 4.3	NA
Mean gestational (± SD) week	9.8 ± 2.7	8.7 ± 3.4	NA
18-20 years	12 (11.2%)	2 (2.2%)	0.037
21-30 years	76 (71.0%)	67 (75.3%)	0.252
> 30 years	19 (17.8%)	20 (22.5%)	0.397
Marital status			
Cohabiting	89 (83.2%)	75 (84.3%)	0.412
Without mate	18 (16.8%)	14 (15.7%)	0.432
History of smoking	3 (2.8%)	1 (1.1%)	0.201
Age of sexual onset			
≤ 16 years	12 (11.2%)	8 (9.0%)	0.198
17-20 years	43 (40.2%)	25 (28.1%)	0.091
> 20 years	52 (48.6%)	56 (62.9%)	0.154
Clinical Conditions			
Vaginal discharge	71 (66.4%)	23 (25.8%)	0.019
Vaginal itching	89 (83.2%)	31 (34.8%)	0.016
Pelvic pain	41 (38.3%)	9 (10.1%)	0.034
Dysuria	29 (27.1%)	7 (7.9%)	0.043
Urinary tract infection	52 (48.6%)	14 (15.7%)	0.012
Irregular menstrual cycle	35 (32.7%)	12 (13.5%)	0.017

pelvic pain (Odds Ratio [OR] = 1.53; 95% CI = 1.14-2.08; $p = 0.009$), and *Mycoplasma* infection (OR = 0.44; 95% CI = 0.21-0.93; $p = 0.031$) were independent predictors of RSA (Table 4).

Discussion

About 50% of all pregnant women had a bacterial vaginal infection, of these 80% of the infection was caused by *M. hominis* or *U. urealyticum* [11]. The production of mucinase or fumarase enzymes by some bacteria alters the local immune system and leads to an increase in inflammatory response and reproductive tract infection [12]. Other enzymes such as lipoids and protease produced by some bacteria dissolve the protein and lipid components of Cowl's membrane and lead to spontaneous abortion [13]. Other bacteria then can invade the embryonic tissue and cowl fragility, deciduitis, chorioamnionitis, and abortion [14].

In the present study, a significantly higher ($p = 0.012$) number of patients (48.6%) in the study group were positive for *Mycoplasma* sp., compared to those in the control (14.6%) group. Similar to the present study Cao et al. [15] who assessed the relationship between RSA and mycoplasma infection reported that the presence of *Mycoplasma* infection was significantly higher in the observation group compared to the control group ($p < 0.05$). The study reported *M. hominis* in 33.3% of the chorion and 34.8% of the decidual tissue samples in the observation group. The study also

reported *U. urealyticum* in 44.7% of the chorion and 39.4% of the decidual tissue samples in the observation group [15]. In the present study, *M. hominis* was present in 29.9% of cases and *U. urealyticum* was present in 18.7% of cases, which was lower than that reported by Cao et al. [15]. Lliopoulou et al. [16] who analyzed placental tissue samples from 59 miscarriages, reported that only 3.6% of their samples were positive for *M. hominis*, which was much lower than that reported in the present study [16]. Contini et al. [17] investigated the association between silent bacterial infection and spontaneous miscarriage. The study reported that *M. hominis* and *U. urealyticum* were present in 5% and 3% of the chorionic villi samples, which was much lower than that reported in the present study [17]. Farhadifar et al. [18] and Ramazanzadeh et al. [19] reported much lower rate of *M. hominis*. These studies evaluated the association between *M. hominis* and spontaneous abortion in 109 women. Of the 109 women's endocervical swabs, only 2 (1.83%) samples were positive for *M. hominis* [18,19]. Kataoka et al. [20] evaluated the relationship between vaginal colonization of *Mycoplasma* sp. and preterm birth in 21 women with spontaneous abortion or preterm birth at <34 weeks of gestation. Of these, vaginal colonization of *M. hominis* was detected in 4 (19%) women and *U. urealyticum* in one (4.8%) women and *U. parvum* in 16 (76.2%) women [20].

Table 2. Distribution of *M. hominis* and *U. urealyticum*.

Microorganism	Study group (n = 107)	Control group (n = 89)	p-value
<i>M. hominis</i>	32 (29.9%)	8 (9.0%)	0.024
<i>U. urealyticum</i>	20 (18.7%)	5 (5.6%)	0.015
<i>M. hominis</i> + <i>U. urealyticum</i>	15 (14.0%)	1 (1.1%)	0.032
Negative	55 (51.4%)	76 (85.4%)	0.046

Table 3. Distribution of Mycoplasmas in patients with clinical conditions.

Clinical Conditions	Study group	Control group	p-value
Vaginal discharge	21/71 (29.6%)	4/23 (17.4%)	0.064
Vaginal itching	15/89 (16.9%)	5/31 (16.1%)	0.894
Pelvic pain	17/41 (41.5%)	2/9 (22.2%)	0.027
Dysuria	16/29 (55.2%)	1/7 (14.3%)	0.012
Urinary tract infection	22/52 (42.3%)	7/14 (50%)	0.131
Irregular menstrual cycle	9/35 (25.7%)	0/12 (0%)	NA

Table 4. Multivariate analysis of risk factors for RSA.

Clinical Conditions	ODDs Ratio (OR)	95% Confidence Interval (CI)	p-value
Vaginal discharge	0.81	0.58-1.14	0.254
Vaginal itching	1.37	0.97-1.85	0.067
Pelvic pain	1.53	1.14-2.08	0.009
Dysuria	0.72	0.51-1.04	0.054
Urinary tract infection	1.02	0.61-1.23	0.215
Irregular menstrual cycle	1.00	1.00-1.00	0.121
<i>Mycoplasma</i> infection	0.44	0.21-0.93	0.031

The presence of *U. urealyticum* leads to the metabolism of urea to generate ammonia disturbing the acidic genital tract environment and producing favorable conditions for bacterial infection of the uterine cavity and the fetus [21]. *Mycoplasma* and bacterial infections increase the occurrence rate of pregnancy complications, spontaneous abortion, premature rupture of membrane and premature delivery [22,23]. The higher presence of *M. hominis* and *U. urealyticum* in the present study among RSA patients reveal a possible association between the presence of microorganisms and RSA which was in agreement with other studies [15,16]. In a review article, Cappoccia *et al.* [8] analyzed 22 studies that compared the association of mycoplasmas/ureaplasmas and adverse pregnancy outcome. The review reported that 15/22 studies and 6/11 studies showed a significant association between *U. urealyticum* and *M. hominis*, respectively [8]. Larsen and Hwang [24] reported a putative association between Mycoplasma infection and early pregnancy loss [24]. In contrast, Kataoka *et al.* [20] reported that *U. parvum* not *U. urealyticum* colonization in vagina was associated with late abortion or early preterm birth [20]. Farhadifar *et al.* [18] concluded that *M. hominis* was present in the vaginal tract of pregnant women but was not associated with spontaneous abortion [18].

In the present study, pelvic pain (OR = 1.53; 95% CI = 1.14-2.08; $p = 0.009$) and *Mycoplasma* infections (OR = 0.44; 95% CI = 0.21-0.93; $p = 0.031$) were independent predictors of RSA. There was a high prevalence of the *M. hominis*/*U. urealyticum* and multivariate analysis revealed Mycoplasma infections as a significant predictor of RSA. To the best of our knowledge, this is the first study that analyzed the predictors of RSA, hence there were no studies to compare this data. Further studies with a larger population are warranted to validate our findings.

Conclusions

There was a high prevalence of the *M. hominis*/*U. urealyticum*, the relationship between *Mycoplasma* infection and RSA was significant after controlling for clinical conditions. Pelvic pain and *Mycoplasma* infections are the independent predictors of RSA.

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Authors' contributions

Study concept and design: Jian Li, Peijun Li; Acquisition of data: Jian Li, Yuecheng Hu; Analysis and interpretation of data: Jian Li, Yuecheng Hu; Drafting of the manuscript: Jian Li, Yuecheng Hu; Critical revision of the manuscript for important intellectual content: Yuecheng Hu, Peijun Li; Statistical analysis: Yuecheng Hu; Study supervision: Peijun Li.

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