Original Article

Association of *Mycoplasma genitalium* with infertility in North Indian women

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Abstract

Objectives: Data regarding the association of *Mycoplasma genitalium* with infertility is scarce. This study was planned to look for the presence and association of *M. genitalium* in women with infertility. **Materials and Methods:** A prospective observational study was conducted on 100 cases of infertile women. The control group included 100 healthy fertile women. Samples of first void urine (FVU), endocervical swabs (ECS), and endometrial biopsies were subjected to polymerase chain reaction targeting *MgPa* gene to look for the presence of *M. genitalium* DNA. All endometrial biopsy samples were subjected to histopathological examination. A detailed clinical history of patients was taken, and all relevant investigations were recorded. **Results:** *M. genitalium* was found in 16% of women with infertility from either of the samples that is, FVU and/or ECS and/or endometrium biopsy, and none from controls. ECS and biopsy could detect the highest number of cases (27%). Asymptomatic cases predominated in the study and *M. genitalium* positivity (73.3%) was seen more in primary infertility. Tubal occlusion and disordered proliferative endometrium were demonstrated in 33% and 26.66% of *M. genitalium* positive cases respectively. **Conclusions:** The study shows an association of *M. genitalium* infection and infertility and suggests routine screening of this pathogen in patients with infertility.

Key words: India, infertility, *Mycoplasma genitalium,* polymerase chain reaction

INTRODUCTION

Mycoplasma genitalium, first isolated in 1981 is an important cause of nongonococcal urethritis (NGU) in men.[1] Evidence has shown that *M. genitalium* is sexually transmitted with a prevalence rate of 56% among male sexual partners of infected women and 32% among female partners of infected men with NGU.[2,3] Moreover, studies have indicated *M. genitalium* as an emerging cause of female genital tract disease.[4] Detection of *M. genitalium* is challenging as the organism is fastidious and has a long turnaround time for culture, taking months (8 weeks or more) to grow. Nucleic acid amplification tests (NAAT) are the only available diagnostic tools to identify *M. genitalium*.

Polymerase chain reaction (PCR) based studies have shown that *M. genitalium* is associated with female upper and lower genital tract disease that is, mucopurulent cervicitis, as well as endometritis, pelvic inflammatory disease (PID), and salpingitis.[2,5-8] *M. genitalium*'s adherence to fallopian tube mucosal epithelial cells has been demonstrated in organ culture.[9] In addition, serological studies have revealed a strong association between prior *M. genitalium* infection and tubal

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factor infertility (TFI).[10] Serological studies are rather evidence for recent or long-term infections while NAAT studies show a link between ongoing infection and infertility.

Infertility is a growing problem and in many instances the etiological factor, particularly of infectious origin cannot be determined. Mycoplasmas can be easily overlooked as they cause low grade asymptomatic infections and there is a lack of sufficient laboratory infrastructure for diagnosing mycoplasmas in most of the hospitals in developing countries. Also, there is a lack of awareness among physicians about *M. genitalium* infection. There is sparse literature showing the link of *M. genitalium* with infertility with no report from India. This study was hence planned to look for the presence of *M. genitalium* in women with infertility and to investigate the causal relationship between *M. genitalium* and infertility.

MATERIALS AND METHODS

A total of 100 women with infertility who had normal montoux test, normal chest X-ray, normal hormonal level, and husband having normal semenogram attending the Infertility Clinic of Gynaecology out patient's department (OPD) of our tertiary care hospital in the North India were enrolled in the study. Infertility was defined according to the operational definition proposed by the World Health Organization as 1-year of unprotected intercourse without pregnancy, which may again be primary or secondary. Primary infertility was defined as inability of the female to conceive inspite of regular sexual intercourse for two years while secondary infertility was referred to when the female had conceived earlier but failed to do so subsequently inspite of regular exposure to sexual activity for two years.[11] Tubal infertility was said to be present if hydrosalpinx was seen on hysterosalpingography. Exposure to sexual activity for breastfeeding female was calculated after lactational amenorrhoea was over. All the patients on antibiotic therapy in previous 2 months were excluded from the study. One hundred healthy fertile women who had delivered recently and were attending the OPD for post natal checkup and contraceptive advice were included as controls. A detailed clinical history of the patients was taken, and all relevant investigations were recorded. A written informed consent was taken from all the patients, and the study was approved by the Institute Ethics Committee. All the specimens were blinded throughout the duration of laboratory investigations, until prior to the data analysis.

First void urine (FVU), three endocervical swabs (ECS) and endometrial biopsy samples were collected from the study group whereas only FVU and endocervical swabs were taken from the control group under strict aseptic precautions. FVU was collected and transported in a sterile plastic container and was stored at -20°C till further use. Endocervical swabs were collected from all the patients using thin Dacron swabs after thorough cleaning of the introitus with sterile normal saline. Endometrial biopsy was obtained using a curette under strict aseptic conditions under sedation with or without paracervical block. The samples were collected in normal saline for culture and in formalin for histopathological examination. The samples were transferred to laboratory in SP4 transport medium.

For *Neisseria gonorrhoeae,* modified New York City medium and for *Mycobacterium tuberculosis*, Lowenstein–Jensen medium was used for inoculation of samples. Culture of genital *Mycoplasma* was done by using pleuropneumonia like organism broth.[12] The reference strains of *Ureaplasma urealyticum* (NCTC 10177) and *Mycoplasma hominis* (NCTC10111) were used as positive controls. For *Chlamydia trachomatis* detection, PCR targeting 368 bp gene encoding endonuclease protein belonging to Phospholipase D superfamily was carried out.[13] Samples positive for *C. trachomatis, M. hominis* and *U. urealyticum* were excluded from the study.

All the samples were subjected to PCR amplification to look for the presence of *M. genitalium* by targeting *MgPa* gene as described previously by Jensen *et al*.[14,15] Briefly, 1.8 ml of urine was centrifuged at 20,000 × *g* for 15 min. The supernatant was discarded. Tubes were left for at least 1 min to let the remaining urine settle down at the bottom. Again the supernatant was pipetted out and 300 μl of Chelex 100 slurry (Sigma, USA) (20% w/v Chelex 100 in Tris-EDTA buffer [10 mmol/l Tris-HCl, pH 8.0]) was added. The mixture was vortexed vigorously for 60 s and then incubated at 95°C for 15 min. After a brief centrifugation, 10 μl of the supernatant was used for PCR.

Endocervical sample preparation for the *M. genitalium* PCR was performed by centrifuging 250 μl of the specimen in SP-4 medium at 30,000 × *g* for 15 min, resuspending the pellet in 50 μl lysis buffer with 200 μg proteinase K/ml, incubating at 55°C for 30 min, and at 94°C for 10 min. After heat treatment, 50 μl specimen diluent was added. The primers used (Sigma Aldrich Chemicals, USA) were: Forward primer (*MgPa* F) – 5'

AGT TGA TGA AAC CTT AAC CCC TTG G 3', and Reverse Primer (*MgPa* R) – 5' CCG TTG AGG GGT TTT CCA TTT TTG C 3'.

A final reaction volume of 100 μl containing × 1 PCR buffer (10 mM Tris-HCI [pH 9.0], 50 mM KCl) with 4.5 mM $MgCl_2$, 0.2 μM of each primer, 1U of Taq polymerase (Roche Diagnostics, Germany), 125 μM concentration of dNTP mix (Roche Diagnostics, Germany), and 50 μl of DNA extract was made. PCR was performed in a thermal cycler (Eppendorf, Germany) beginning with an initial denaturation at 95°C for 5 min, followed by a total of 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 67°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 6 min. All the batches of PCR were run along with a positive control using *M. genitalium* DNA obtained from Statens Serum Institute, Denmark and a negative control containing all the reagents without DNA. The amplicons were visualized after electrophoresis on 2% of agarose gel stained by ethidium bromide and examined under ultraviolet transillumination for a 281 bp product. All the PCR positive were further confirmed with commercially available PCR kit (SORPOLINE™ *M. genitalium* End Point PCR Kit, Lithuania) for the detection of *M. genitalium*. The strict physical separation between samples was maintained to avoid any PCR product carryover. Endometrial biopsy preparation for PCR also was carried in a similar manner like that of the endocervical samples except that biopsy samples were minced before processing.

A part of endometrial biopsy samples was subjected to HPE which was carried out according to standard methods.

Chi-square test and Fisher's exact test were used for analyzing the results. The data were analyzed using statistical software SPSS software 15.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Of the total 100 cases, *C. trachomatis* DNA was detected in six infertility patients negative for *M. genitalium*; these cases were excluded from the study. No other pathogen tested was detected in any of the specimens. Therefore, the infertility group included 94 patients. Of these 94 women, the majority were in age groups of 20–30 years. The mean age of the women was 29.08 years (±3.34) in the infertility group and 27.30 years (±3.32) in the controls. Overall, *M. genitalium* was detected in 16% (15/94) of women with infertility from either of the samples that is, FVU and/or ECS and/or endometrial biopsy. Most of *M. genitalium* positive women (60%) were from a rural background. Of 15, PCR positive *M. genitalium* cases, ECS, and biopsy could detect a maximum of 26.6% of the cases followed by ECS alone in 20%, endometrial biopsy alone in 20%, urine alone in 13%, and urine and ECS in 13% [Table 1]. All the three samples (urine, ECS and biopsy) were positive only in 6.6% of the patients. In the control group, none of the 100 women was *M. genitalium* positive from urine or ECS ($P = 0.00003$). The association between *M. genitalium* and infertility was highly significant whether the organism was detected in the cervix, urine, or endometrial biopsy or in a combination of either of the sample. *M. genitalium* was found to be infecting the patients without any coinfection with other pathogens tested. Asymptomatic cases ($n = 77$) predominated in the study. Of these asymptomatic patients, 51 (66.23%) had primary infertility while the rest had secondary infertility (33.76%). Among the 17 (21%) symptomatic patients, there were 11 (64.7%) cases of primary infertility and 6 (35.29%) cases of secondary infertility. Of the 15 women who were found to be positive for *M. genitalium* infection, 11 (73%) had primary infertility, while remaining 4 (26%) had secondary infertility ($P < 0.001$) [Table 2].

The most common symptom with infertility was discharge which was present in 12 (57%) patients, followed by burning micturition 7 (33%), scanty

Table 1: Distribution of *M. genitalium* PCR results according to specimens

Patients (*n*=94)				Controls* (*n*=100)
FVU	ECS	Endometrial biopsy	Total positives=15 (16%)	Total positives=0 (%)
+	–	–	2 (13)	0 (0)
–	+	–	3 (20)	0 (0)
–	+	+	4 (26.6)	0 (0)
+	+	–	2 (13)	0 (0)
+	+	+	1 (6.6)	0 (0)
–	–	+	3 (20)	0 (0)
			P=0.00003	

*Controls were not subjected to endometrial biopsy. ECS=Endocervical swab; FVU=First void urine; PCR=Polymerase chain reaction; *M. genitalium=Mycoplasma genitalium*

Table 2: *M. genitalium* PCR positive cases and type of infertility

Type of infertility	*M. genitalium* infection (15)
Primary infertility (62) (%)	11 (73.3)
Asymptomatic 51 (66.23)	8 (53.3)
Symptomatic 11 (64.7)	3 (20)
Secondary infertility (32) (%)	4 (26.67)
Asymptomatic 26 (33.76)	3 (20)
Symptomatic 6 (35.29)	1 (6.67)

PCR=Polymerase chain reaction; *M. genitalium=Mycoplasma genitalium*

menses 1 (4.7%), and menorrhagia (4.7%). Amongst the women with *M. genitalium* infection, 40% (6/15) had history of PID as compared to 10.2% (9/88) in those without infection, and the association was statistically significant ($P < 0.001$) [Table 3]. Tubal occlusion was seen in 5 (33.3%) women with *M. genitalium* infection, and none of the women negative for *M. genitalium* infection had tubal occlusion ($P < 0.001$). All these five women were asymptomatic. *M. genitalium* positivity was correlated with histopathological findings, and abnormal findings which included disordered proliferative endometrium were observed in four patients of *M. genitalium* positive cases. However, no statistical significant difference was seen between abnormal findings in HPE and patients' positivity or negativity for *M. genitalium* by PCR.

Table 3: Comparison of different parameters of *M. genitalium* PCR positive and negative women

Parameters	*M. genitalium* positive (*n*=15) (%)	*M. genitalium* negative (*n*=79) (%)
Age	29.08 (±3.34)	27.30 (±3.32)
Socioeconomic status		
Urban	6 (40)	33 (38.8)
Rural	9 (60)	52 (61.1)
History of PID	6 (40)	9 (10.2)
Abnormal HPE findings	4 (26.6)	17 (20)
Tubal occlusion	5 (33)	0 (0)
	P<0.001	

HPE=Histopathological examination; PID=Pelvic inflammatory disease; PCR=Polymerase chain reaction; *M. genitalium*=*Mycoplasma genitalium*

DISCUSSION

Infertility is an emerging health problem in many countries of the world including India. In many instances of asymptomatic or oligosymptomatic infections in the patients with infertility, it is difficult to find the etiological cause. *M. genitalium* can be a cause of such asymptomatic infections and can be easily overlooked by clinicians. In our study, *M. genitalium* was found in 16% of infertile women. The higher detection may be due to the fact that three different types of samples (FVU, ECS and endometrium biopsy) were collected in this study. This is in agreement with the study by Jensen *et al.* which demonstrated that for the optimal sensitivity of detection of *M. genitalium*, two samples should be taken from women.[16] We found that endometrial biopsy supplemented with cervical swab could detect a maximum number of cases (27%). Another study in 51 infertility patients and 23 healthy fertile women reported high prevalence of *M. genitalium* in the cervical canal by PCR (20% in cases verses 4% in controls), however the results were not statistically significant.[17]

Most of the PCR positive *M. genitalium* patients were asymptomatic in our population (i.e. 81.9% [77/94] asymptomatic vs. 18% [17/94] symptomatic). The role of asymptomatic infection with genital *Mycoplasma*, particularly *M. genitalium* is known in cases of infertility caused due to fallopian tube disorders. Clausen *et al.* found that anti-*M. genitalium* antibodies were detected in 22% of women with TFI, whereas in the control group of women with unobstructed fallopian tubes, antibodies were found only in 6% of patients.[18] Thus, infection with *M. genitalium* is an independent risk factor in the development of an inflammatory process damaging the fallopian tubes causing infertility. In our study, tubal occlusion was observed in 33% of *M. genitalium* positive patients and none in *M. genitalium* negative patients. Moreover, 40% of the women in our study had a history of PID, which was found to be statistically significant ($P < 0.001$). In analysis of 586 women in a study which analyzed the association between *M. genitalium* and reproductive morbidity among population of women presenting with signs and symptoms of PID (PEACH study), the rates of sequelae were found to be high for infertility that is, 22%. These findings suggest causal relationship between *M. genitalium* infection and infertility, and that the detection of this particular organism is often associated with the disease and can cause permanent damage to the reproductive tract when there is chronic infection, although the symptoms are very mild.[5,19,20]

We observed that the incidence of *M. genitalium* was more common in women with primary infertility as compared to secondary infertility though this finding was not statistically significant. Surprisingly, we observed that *M. genitalium* was detected in our patients without any co-infection with other common pathogens. This further suggests that *M. genitalium* infection could be the primary pathogen responsible for infertility. The study also demonstrated that more than one sample that is, urine and ECS and/or endometrial biopsy are needed to increase the detection rate of *M. genitalium* in infertility patients. However, the studies with larger sample size are needed to further elucidate the extent and severity of infertility caused by *M. genitalium*.

C. trachomatis had low positivity of 0.6% in the present study. A study from Aligarh, India has shown the presence of *C. trachomatis* in 28.1% of infertile women while, in another study, anti-chlamydial IgG antibodies were detected in 68% of women with infertility.[21,22] The low detection of this pathogen in our study may be due to our center being a tertiary care center and patients

referred here are already exposed to many antibiotics including doxycycline and azithromycin to which *C. trachomatis* is sensitive.

The detection of *M. genitalium* infection in infertile couples is still not included in the standard tests performed for infertility because there is scanty evidence of association of *M. genitalium* with infertility; also, there is lack of awareness among clinicians regarding infertility due to *M. genitalium* infection. The present study clearly indicates the link between *M. genitalium* infection and infertility and our findings strongly suggest that clinicians should raise their index of suspicion for *M. genitalium* infection as an etiological agent when treating infertility and such patients should be routinely screened for *M. genitalium* even if they are not symptomatic.

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Conflicts of interest
There are no conflicts of interest.

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