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## Original Article

# Mycoplasmatecae species are not found in Fallopian tubes of women with tubo-peritoneal infertility

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### A B S T R A C T

**Background:** The role of mycoplasmas on the development and sequelae of pelvic inflammatory disease remains controversial. The objective of the present study is to correlate directly the presence of *Mycoplasmatecae* through polimerase chain reaction (PCR) determinations in cervix and Fallopian tubes of infertile patients with tubo-peritoneal factor diagnosed through laparoscopy.

**Methods:** Thirty patients with tubo-peritoneal infertility and 30 normal fertile patients were included in the study; cervical samples and tubal flushings were obtained during laparoscopy. PCR determinations for the detection of genetic material of *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma urealiticum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* in cervix and tubal flushings were performed.

**Results:** No *Mycoplasmatecae* species as "only" microorganisms were found in tubal flushings of tubo-peritoneal infertility patients, whereas three (10%) fertile patients with normal tubes were positive for mycoplasma presence. This difference was not significant ( $p = 0.237$ ). Among the 30 patients suffering from tubal infertility diagnosed through laparoscopy, *Mycoplasmatecae* species were not detected in the Fallopian tubes by PCR determinations, while in normal tubes from fertile patients these and other microorganisms could be found without distorting tubal anatomy.

**Conclusion:** *Mycoplasmatecae* species were not detected in Fallopian tubes of women with tubo-peritoneal infertility.

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## Introduction

Tubal and peritoneal factors are important causes of infertility among couples investigated for this condition affecting their

reproductive potential, and pelvic inflammatory disease is often the origin of these altered factors.<sup>1-4</sup>

*Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) are bacteria that have been clearly identified as etiologic agents of pelvic inflammation causing infertility.<sup>4</sup> Nevertheless, in recent

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years, *Mycoplasma* and *Ureaplasma*, two genera belonging to the family mycoplasmataceae, order Mycoplasmatales, class Mollicutes, have been proposed as causatives of genitourinary disease in both men and women. Both microorganisms, often called generically mycoplasmas, are characterized by the lack of cell wall, being the smallest prokaryotic organisms with self-replication capability. Of the various Mycoplasmataceae that have been detected in the genitourinary tract, *Mycoplasma hominis* (MH), *Ureaplasma urealyticum* (UU), and *Mycoplasma genitalium* (MG) have been associated with disease; of importance is the fact that MH and UU are frequently isolated from the lower urogenital tract of healthy adults, men and women, but the presence of MG in the genital tract of healthy people is less frequently documented and therefore has been strongly correlated with disease.<sup>5</sup> Moreover, experimental animal models show that direct inoculation with MG can cause upper tract inflammatory disease.<sup>6,7</sup> Of importance, is the fact that culture isolation is not clinically practical for MG, so the presence of this microorganism is mainly determined through polymerase chain reaction (PCR),<sup>8</sup> or indirectly through serum antibodies.<sup>9-11</sup> MG appears to be an increasing agent of sexually transmitted diseases.

The question if the above mentioned Mycoplasmataceae are the cause of tubal and/or peritoneal infertility has been studied mostly indirectly, mainly through serological determinations, even though these microorganisms have been isolated from the cervix, endometrium, recto uterine pouch, and in one case from acute salpingitis.<sup>5</sup>

The objective of the present study was to correlate directly the presence of these Mycoplasmataceae through PCR determinations in cervix and Fallopian tubes of infertile patients in which a tubo-peritoneal factor was diagnosed through a laparoscopic procedure.

## Material and methods

This is a prospective case-control study conducted between April 2008 and May 2010 at the Complejo Hospitalario San Jose, Santiago, Chile, a hospital associated to the Facultad de Ciencias Medicas of the Universidad de Santiago de Chile. During this period, 30 infertile women, with a mean age of  $33 \pm 5$  years (range 24-41), in which a laparoscopy, performed as part of their infertility investigation, revealed a tubal abnormality (tubal fimosis, hydrosalpinx, and/or periamputar or fimbrial tubal adhesions) were included in this study (tubo-peritoneal infertility). Patients with a history of antibiotic treatment in the previous three months before laparoscopy, and also those in which tubal or peritubal endometriosis was observed were excluded from this study. Thirty patients, mean age  $36 \pm 5$  years, (range 27-47), undergoing laparoscopic tubal ligation, and in which normal tubes were identified, were included as controls. This study was approved by the Ethics Committee and an informed consent was obtained before the procedure.

### Material sampling

An intracervical sample was taken immediately before laparoscopy by rotating a rayon sterile swab (Copan, Italy).

During laparoscopy, the tubes were flushed with 2-4 mL 9%-saline solution, immediately after the laparoscope and auxiliary instruments were introduced. The tubes were flushed by gently grasping the tubal ampulla near the fimbrial portion with an atraumatic tubal forceps and introducing an epidural catheter (BD Perisafe I® – Mexico, Cal. 19 G) inside the ampulla through the abdominal tubal ostium via a suprapubic puncture with a BD® Tuohy type needle Cal. 16 G, 88.9 mm long.

The cervical and tubal samples were stored in sterile tubes containing SP2 transport media, at 4°C for a maximum of two hours before being frozen and stored at -20°C.

### Sample preparation

In the present study CT, NG, MG, MH, UU and *Trichomonas vaginalis* (TV) were assayed by duplex PCR in cervical and Fallopian tube flushings from women with laparoscopically-confirmed Fallopian tube damage, and from healthy controls.

### DNA extraction

Genomic DNA for the multiplex PCR assays was prepared from clinical specimens using a Favorgen genomic DNA extraction kit (Ping Tung – Taiwan) in accordance with the manufacturer's instructions. The extracted DNA samples (undiluted) were measured using a spectrophotometer (SmartSpec 3000; BioRad) at 260 nm and the correct DNA dilution for PCR was calculated.

### Multiplex PCR method

A multiplex PCR assay for simultaneous detection of the six pathogens was performed using the DPO-based Seeplex STD detection assay according to the manufacturer's (Seegene) instructions. The target genes for multiplex PCR were as follow: the DnaB-like protein gene on the cryptic plasmid of CT, the porin protein A (*porA*) gene (pseudogene) of NG, the DNA gyrase (*gyrA*) gene of MG, the glyceraldehyde 3-phosphate dehydrogenase (*gap*) gene of MH, the urease (*ureG-D*) gene of UU, and the  $\beta$ -tubulin 1 (*btub1*) gene of TV. The kit includes amplification of the Arabidopsis cellulose synthase (*CesA3*) gene as an internal control (IC), which is designed to detect the presence of PCR inhibitors. The *CesA3* gene was amplified with a specific primer pair (forward: 5'-GCATCTTCTTAGCCATCCCAAGAIIIIICTCTTCGTCT-3' and reverse: 5'-CAAGCCGCGAGCATAATAACCATIIIIIAAGGATTGATCC-3').

Amplified fragments were separated by agarose gel electrophoresis (agarose, 2%) and identified by SYBR Safe DNA gel stain (Invitrogen, Life Technologies – Carlsbad, California, USA).

### Statistical analysis

Statistical analysis was performed using the STATA (Statacorp) statistical software, version 11.1. Analysis of the distribution of discrete parameters was performed using Fisher's exact test. A p-value < 0.05 was considered statistically significant.

## Results

Positive PCR determinations for detection of genetic material of *Mycoplasmataceae* species (MH, MG, and UU) and three other microorganisms (NG, CT and TV) obtained from samples of both endocervix and Fallopian tubes flushings from 60 patients, 30 of them with a tubo-peritoneal infertility factor diagnosed through laparoscopy, and 30 fertile patients with normal tubes and peritoneum at laparoscopy are detailed in Table 1. Twenty (33.33%) of the 60 women were infected with at least one pathogen in cervix or Fallopian tube samples, or both.

The presence of  $\geq 1$  of any microorganism in one or both investigated sites of the genital tract according to tubo-peritoneal status was more frequent in the control group (40%) than in case patients (26.7%), but this difference (Table 2) did not reach statistical significance ( $p = 0.412$ ). Also, when mycoplasma-only detection in both sites of the female genital tract was compared, no significant difference between normal patients (16,6%) and tubo-peritoneal infertility patients (10%) was observed ( $p = 0.706$ ).

When comparing frequency of positive PCR determinations for detection of  $\geq 1$  microorganisms in FT flushings during laparoscopy, a significant difference (26.7%) for fertile patients with normal tubes versus infertile patients with altered tubo-peritoneal factor, ( $p = 0.005$ ) was observed. No *Mycoplasmataceae* species as only microorganisms were found in tubal flushings of tubo-peritoneal infertility patients, whereas three (10%) fertile patients with normal tubes were positive for mycoplasma presence. This difference was not significant (Table 3). No genetic material from any studied microorganism was found in the tubal flushings of any of the 30 infertile patients in which a tubo-peritoneal factor was found at laparoscopy. Noteworthy, MG was not found in any tubal flushing from the 60 patients of this study, and only one determination for this bacteria was found to be positive in the cervix of woman from the tubo-peritoneal factor infertility group.

Finally when comparing presence of mycoplasma-only and presence of  $\geq 1$  microorganism in the cervix, no difference was found between case and control groups (Table 4).

**Table 1 - Presence of microorganisms in the female genital tract according to tubal status**

Tubo-peritoneal infertility			Control (normal tubes)		
Patient	Fallopian Tube	Endocervix	Patient	Fallopian Tube	Endocervix
1	(-)	CT + MG	1	NG	NG
2	(-)	MH	2	NG + UU	UU
3	(-)	MH	3	UU	UU
4	(-)	NG	4	NG	NG + TV + MH
5	(-)	NG	5	NG + CT	(-)
6	(-)	NG	6	NG + UU	(-)
7	(-)	TV	7	MH	(-)
8	(-)	UU	8	UU	(-)
			9	(-)	NG
			10	(-)	UU
			11	(-)	NG + UU
			12	(-)	MH

MH, *Mycoplasma hominis*; UU, *Ureaplasma urealyticum*; MG, *Mycoplasma genitalium*; NG, *Neisseria gonorrhoeae*; CT, *Chlamydia trachomatis*; TV, *Trichomona vaginalis*.

**Table 2 - Microorganisms in female genital tract according to tubal status assessed by laparoscopy**

		Tubo-peritoneal infertility	Control (normal tubes)	p-value*
Presence $\geq 1$ microorganism in female genital tract	n (%)	8 (26.7%)	12 (40%)	0.412
Micoplasmas-only in female genital tract	n (%)	3 (10%)	5 (16.6%)	0.706
Total	n (%)	30 (100%)	30 (100%)	

\*p-values were obtained by Fisher test.

**Table 3 - Presence of microorganisms in FT according to tubal status**

		Tubo-peritoneal infertility	Control (normal tubes)	p-value*
Mycoplasmas-only in FT	n (%)	0 (0%)	3(10%)	0.237
Presence ≥ 1 microorganism in FT	n (%)	0 (0%)	8 (26.7%)	0.005
Total	n (%)	30 (100%)	30 (100%)	

FT, Fallopian tubes; \*p-values were obtained by Fisher test.

**Table 4 - Presence of microorganisms in cervix according to tubal status**

		Tubo-peritoneal infertility	Control (normal tubes)	p-value*
Mycoplasmas-only in cervix	n (%)	3 (10%)	4(13.3%)	0.5000
Presence ≥ 1 microorganism in cervix	n (%)	8 (26.7%)	8 (26.7%)	0.6145
Total	n (%)	30 (100%)	30 (100%)	

\*p-values were obtained by Fisher test.

## Discussion

Altered tubo-peritoneal factors are a frequent cause of infertility. Pelvic inflammatory disease is one of the most important pathological conditions that cause permanent damage and adhesions affecting the Fallopian tubes.<sup>1-4</sup> There is also sufficient evidence that most women suffering from tubal infertility have no prior history of symptomatic pelvic inflammatory disease, and that lower genital tract infections are related to infertility.<sup>12</sup>

For several years it has been proposed that *Neisseria gonorrhoeae* and *Chlamydia trachomatis* were the most important pathogens with potential ability for inducing irreversible damage in the Fallopian tube mucosa.<sup>4</sup> Nevertheless, in the last decades, with increased frequency, several studies have associated Mycoplasmatraceae infections with endometrial and tubal damage.<sup>8,13-15</sup>

There is only one study that correlates the direct presence of a Mycoplasmatraceae family microorganism (*Mycoplasma genitalium*) with acute salpingitis,<sup>14</sup> and another exploring the occurrence of this bacteria in the recto uterine pouch,<sup>13</sup> but to the authors' knowledge there are no published studies exploring the direct association of PCR confirmed mycoplasma detection inside a Fallopian tube by and tubal damage diagnosed by laparoscopy. Most published studies refer only to indirect detections for assessing past tubal infections.<sup>10,11</sup>

For pathogen detection a multiple duplex PCR system that allows the detection of six pathogens in the same reaction with a sensitivity of ten copies was used, giving maximum detection sensitivity as compared to both culture and detection by conventional PCR. In this way the presence of other pathogens possibly responsible for causing tubal damage could be detected. Thus, the present results could express a mycoplasma-only association with such observed pathology.

Sixty endocervical and tubal samples obtained from 30 infertile patients with a tubo-peritoneal factor were analyzed, and 30 from fertile control patients as previously described. Twenty (33,33%) of the 60 women had at least one pathogen infecting cervix or Fallopian tube samples; in the control group 40% (12/30 patients) were infected, as compared to 26.7% (8/30 patients) in the tubo-peritoneal infertility group, but this difference was not significant. Among infected patients, the analysis was also performed considering those who were infected exclusively with *Mycoplasmatraceae* species. Once again a non-significant difference (10%, 3/30) versus (16.6%, 5/30), between cases and controls was found.

Noteworthy is the fact that, considering all studied pathogens (MG, MH, UU, NG and CT) as well as mycoplasmas-only, none of them were found by PCR determinations in tubal flushings of infertile patients with tubal damage; while in fertile patients with normal tubes, all pathogens were considered. This striking finding could be explained by remodeling of the tubal epithelium. The principal feature of salpingitis is the extensive tissue remodeling that produces chronic sequelae such as scars and lumen obstruction. At the cellular level, the infection also involves changes in the local immune response; for example, in damaged Fallopian tubes due to NG infection there are several changes in the expression levels of proteins involved in the host pathogen response, changes that are key for pathogen establishment due to their ability to manipulate the immune response. Normally, the immune system of the female genital tract has several mechanisms to prevent immune responses against gametes and conceptuses. In fact, the female genital tract has an immune privilege, which is to use the FAS-L/FAS-R system to induce apoptosis of activated lymphocytes in the lumen of the genital tract, preventing their return to the lymph nodes and the assembly of an immune response against antigens.<sup>16,17</sup>

The basis of this system is in the epithelium of the Fallopian tubes. One of the main consequences of sexually transmitted infections is the loss of the epithelium and its replacement by connective tissue. This may be the reason why these pathogens can survive only in healthy tubes, taking advantages of FAS-I/FAS-R to avoid the immune response. In contrast, in the damaged Fallopian tube, the loss of critical components of this system would allow for an immune response that could eliminate the pathogens. This proposition will require further studies.<sup>18</sup>

Perhaps it is impossible to find bacterial growth in the tubes after infection with *Mycoplasma genitalium* or another pathogen that generates changes in morphology<sup>19</sup> or scarring of the Fallopian tube. However, this could be contradictory with the fact that humans can suffer several episodes of infections, and may be reinfected if the damage from a previous infection was partial and did not cause a remodeling or total occlusion of the tube.

Results of one study are contradictory with the detection of *M. genitalium* in salpingitis patients.<sup>20</sup> In this study, an indirect hemagglutination assay (IHA) was performed using sonicated MG cells on sensitized sheep erythrocytes. Forty two patients with acute salpingitis determined by laparoscopy were investigated for antibodies to *M. genitalium*, and none of the patients tested positive. One explanation for this might be that the measurements of antibodies were performed by different methods, IHA and immunoblotting. Another reason for the lack of antibodies to MG could be the relatively small sample size of 42 symptomatic patients tested. Of importance is the fact that MG was detected in only one case, in the cervix of case patient, but this bacteria could not be detected either in the cervix or in the Fallopian tube when other pathogens beside Mycoplasmataceae were excluded. MG was not detected in any control patient either. These results seem to support that this microorganism has a very low incidence in the studied population, and is a striking difference from other studies in other countries,<sup>21,22</sup> in which MG has been found in a significantly higher proportion in infertile patients when compared to fertile controls, and also in patients with idiopathic infertility. MG has been found to alter the tubal epithelium after *in vitro* culture studies, but MH did not seem to produce any morphologic change in this same study.<sup>19</sup>

MH was found in the samples exclusively infected with mycoplasmas, but due to the low number of infected subjects the role in the development of sequelae remains uncertain. When mycoplasmas were detected, UU was more frequently found in both groups, with a significant difference between cases and controls, with a higher incidence among the latter, indicating that UU is able to grow in healthy Fallopian tubes as a commensal microorganism. These results are in agreement with a study that found that UU asymptomatic colonization is associated with immune suppression.<sup>23</sup> However, among cases UU was detected in cervix samples without matching the corresponding Fallopian tube. It has been proposed that asymptomatic colonization may induce a TH1 response in the endometrium, avoiding implantation and development of the conceptus. Another striking possibility is that mycoplasmas might negatively affect only a specific subset of women.

An earlier study concluded that CIAS1 7 with repeated polymorphism increases the likelihood of mycoplasma infection associated with female infertility. Further studies are required to evaluate this observation.

The detection of UU in the control group presented all possible patterns of infection: a patient with detection in the Fallopian tube only, a patient with detection only in the cervix, and a patient with detection in both. From these three possibilities, the most striking was the detection in the Fallopian tube without matching in the cervix. A possibility is that the bacterium is able to evade the difficulties to ascend the genital tract and reach the Fallopian tubes, and having done so, does not have to compete with any other bacteria because the Fallopian tubes are infertile. On the contrary, the bacteria reaching the ectocervix must compete for resources and be compatible with the metabolic wastes from the normal flora of the vagina.

Until now, the role of UU in salpingitis remains controversial. However, the overall rates of infection with UU were higher, indicating the possible role of UU as a co-infecting pathogen. Due to this observation, the percentage of results with mycoplasma-only could be explained by the selective death of the main pathogen, in the case of treatment with certain antibiotics that affect the cell wall not present in mycoplasmas. This possibility requires further study.

The results of this study do allow for a correlation between the presence of Mycoplasmataceae and tubal damage diagnosed by laparoscopy; however, it is not possible to rule out another strain of mycoplasma or a role during the acute phase of salpingitis. Regarding this last point, studies that have shown the correlation between antibodies against MG, without the match detected by PCR method, and women with infertility due to tubal damage independent of other pathogens, validate this inference.<sup>24</sup>

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## Conflict of interest

All authors declare to have no conflict of interest.

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