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Prevalence of genital *Mycoplasma* and response to eradication treatment in patients undergoing assisted reproductive techniques

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ABSTRACT

Introduction. Several studies have reported greater success of fertilisation by ART in couples who were not infected by *Ureaplasma*. Increased semen quality and better results have also been observed in couples who were treated with antibiotics to eradicate the infection. The aim of this study was to determine the prevalence of genital mycoplasmas in urine samples from male partners enrolled in the Assisted Reproduction Program (ARP) in our healthcare area so that, positive cases can be treated prior to the use of ART in order to increase the quality of semen, improve the embryo implantation rates and minimize the risk of adverse effects during pregnancy.

Material and methods. This study included couples enrolled in the ARP during 2016. *Mycoplasma* detection was made using real-time PCR. In positive cases, both members of the couple were treated with antibiotics until eradication of the microorganism. The antibiotics used were: azithromycin, doxycycline, levofloxacin, moxifloxacin, and clindamycin.

Results. Of the 205 men studied, 33 were positive: *Ureaplasma urealyticum* 15.1%, *Mycoplasma hominis* 3.9%. Eradication treatment with azithromycin failed in 50% compared to 10.2% for doxycycline. Of the 5 cases treated with levofloxacin, only 2 achieved elimination of *U. urealyticum*.

Conclusions. We consider that genital mycoplasma routine screening could be useful in order to increase the quality of semen which could simplify the *in vitro* fertilisation procedures and raise the success rate of embryo implantation and pregnancy, especially when fast, sensitive and specific techniques as real time PCR are used.

Keywords: *Mycoplasma*; *Ureaplasma*; Assisted reproduction; doxycycline; azithromycin

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Prevalencia de micoplasmas genitales y respuesta al tratamiento de descolonización en pacientes de reproducción humana asistida

RESUMEN

Introducción. Se han publicado estudios que demuestran mayores tasas de éxito en las técnicas reproducción asistida (TRA) en parejas no infectadas por micoplasmas. El objetivo de este estudio fue determinar la prevalencia de los micoplasmas genitales en muestras de orina del miembro masculino de las parejas incluidas en el Programa de Reproducción Asistida en nuestro Área Sanitaria realizando un tratamiento descolonizador con el fin de incrementar la calidad del semen, mejorar las tasas éxito de la embriotransferencia y minimizar los efectos adversos sobre la gestación.

Material y métodos. Participaron parejas incluidas en el Programa de Reproducción Asistida durante 2016. La detección de los micoplasmas se realizó por PCR en tiempo real. En los casos positivos, la pareja fue tratada con antibióticos hasta la erradicación del microorganismo. Los antibióticos usados fueron: azitromicina, doxiciclina, levofloxacino, moxifloxacino y clindamicina.

Resultados. De los 205 hombres estudiados, 33 fueron positivos: *Ureaplasma urealyticum* 15,1%, *Mycoplasma hominis* 3,9%. Azitromicina fracasó en el 50% de los casos y doxiciclina en el 10,2%. Con levofloxacino solo en 2 de 5 se consiguió la erradicación de *U. urealyticum*.

Conclusiones. El cribado de rutina de los micoplasmas genitales puede ser útil para mejorar la calidad del semen. Esto permitiría simplificar los procedimientos de fertilización *in vitro* e incrementar las tasas de éxito en la implantación de los embriones y en la gestación, especialmente con la aplicación de técnicas diagnósticas rápidas y específicas como la PCR en tiempo real.

Palabras clave: *Mycoplasma*; *Ureaplasma*; reproducción asistida; doxiciclina; azitromicina

INTRODUCTION

Genital mycoplasmas (*Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma genitalium* and *Mycoplasma hominis*) are bacteria found in the placenta and/or embryo in pregnant women. They can cause ascending infection after rupture of membranes and severe problems such as premature membrane rupture, preterm labour, miscarriage, postpartum fever, chorioamnionitis, and infection transmission to the newborn infant [1-5]. Several studies have reported greater success of fertilisation by assisted reproductive techniques (ART) in couples who were not infected by *Ureaplasma* spp. [6, 7]. Increased semen quality and better ART results have also been observed in couples infected by mycoplasmas who were treated with specific antibiotics to eradicate the infection [8, 9]. Moreover, the impact on female reproduction and the health of the fetus and newborn that can have genital mycoplasma suggests a role for routine screening and treatment before undergoing infertility treatment [10-12]. It therefore seems recommendable to avoid the use of semen contaminated/infected with mycoplasmas in ART procedures. However, currently used procedures for selecting and washing spermatozoa prior to fertilisation do not ensure eradication of these bacteria [13].

With the advent of highly sensitive and specific nucleic acid amplification techniques, more accurate estimate of the prevalence of genital mycoplasma as well as the control of the efficacy of the antibiotic treatment can be determined.

This study presents the results obtained after screening for genital mycoplasmas in urine using amplification by real-time polymerase chain reaction (RT-PCR) in patients enrolled in the assisted human reproduction program at University Hospital Complex of Santiago (CHUS), in Santiago de Compostela, Spain.

MATERIALS AND METHODS

Patients. This study included couples enrolled in the assisted human reproduction program carried out by the Assisted Human Reproduction Unit at CHUS during 2016. Couples provided their informed consent after the study was approved by the competent Research Ethics Committee and by the hospital's management (reference number 2015/493). The screening for genital mycoplasmas was performed in male urine. In positive cases, both members of the couple received specific antibiotic therapy and had a post-treatment microbiological test. Couples were asked to use protection (condoms) during sexual intercourse for 7-14 days after initiating the treatment. Both members of the couple had a microbiological test 3-4 weeks after completing the treatment. Couples failing antibiotic treatment were prescribed alternative antibiotics until both members had negative post-therapy microbiological tests. No patient was on antibiotic treatment at least, three months prior to collection of the sample. As part of the study protocol prior to *in vitro* fecundation, all male patients were screened for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in the same urine sample used for mycoplasma detection.

Samples. The first fraction of urine (10-15 mL) was collected from males after at least 2 hours without urinating. Urine was collected under the same conditions for the post-treatment tests in female patients.

Determination of *M. genitalium*, *M. hominis*, *U. urealyticum*, and *U. parvum*. Genital mycoplasma screening was performed using real-time PCR with LightMix *Mycoplasma gen/hom Ureaplasma* (Roche, Switzerland) in accordance with the manufacturer's instructions. Briefly, a 224 or 129 bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (gap) gene for *M. genitalium* or *M. hominis*, respectively, was amplified with specific primers detected with labelled probes, identified by melting curve analysis. In the same way, a 187 bp long fragment of 16S RNA gene was applied for *U. urealyticum*. This kit detects also *U. urealyticum* Biotype 1 (*U. parvum*) according to manufacturer's information. The sensitivity of the test is 10 copies/mL.

Nucleic acids were extracted previously using the Mag-naPure Compact system (Roche, Switzerland) after concentrating the sample by centrifuging 2 mL of urine for 20 minutes at 14,000g [14]. After discarding the supernatant, 200 µL of the re-suspended sediment were used to load the extraction system.

Antibiotic treatment to eradicate genital colonisation. Any positive PCR finding was treated with azithromycin or doxycycline. Quinolones were used when first line treatment failed.

Statistical analysis. All statistical analyses were carried out using SPSS v. 20.0 (IBM Corp., Armonk, NY). Statistical significance was set at $p < 0.05$.

RESULTS

A total of 205 couples were enrolled in the study. The age range was 24 to 54 years in men and 23 to 39 in women, with a median age of 36 years for men and 35 years for women. Most men were Caucasian ($n = 201$). Other ethnic groups were American ($n = 2$) and Gypsy ($n = 2$).

A total of 33 (16.1%) males tested positive by real-time PCR for one or more mycoplasmas: *U. urealyticum* 15.1% ($n = 31$) and *M. hominis* 3.9% ($n = 8$), with 6 (2.9%) cases of coinfection with both microorganisms. No sample proved positive for *M. genitalium*.

Initial treatment for *Ureaplasma* spp. was azithromycin in the first 12 couples studied and doxycycline in the remain-

Table 1 Number of therapeutic failures depending on the antibiotic used for initial treatment in patients positive for *U. urealyticum*

	Men	Women	Couples
Azithromycin	1	8	3
Doxycycline	0	3	1

Table 2 Antibiotics used against *Ureaplasma* spp. and post-treatment real-time PCR success test

1st treatment	2nd treatment	Couple Id.	RT-PCR post-treatment	3rd treatment	RT-PCR post-treatment	4th treatment	RT-PCR post-treatment
Azithromycin	No	9					
		10	Positive	Levofloxacin	Positive	Clindamycin	Negative
		12	Negative				
		25	Negative				
		28	Negative				
		29	Negative				
Azithromycin	Doxycyclin	31	Negative				
		36	Negative				
		39	Negative				
		43	Negative				
		46	Negative				
		48	Negative				
		104	Negative	Moxifloxacin ^a	Negative		
		106	Negative				
Doxycyclin	Levofloxacin	130	Negative				
		159	Positive	Clindamycin	Positive	Clindamycin + probiotic	Positive

^aFor *M. hominis* eradication.

ing 19 couples. Table 1 shows post-treatment test results for the cases positive for *U. urealyticum*. Treatment failed in 16 patients (11 females, 1 male, and 2 couples), including 12 patients treated with a single oral dose of azithromycin 1 g and 4 patients treated with doxycycline 100 mg/12h p.o. for 7 days. One of the couples (couple # 61) who tested negative for *U. urealyticum* after initial treatment with doxycycline remained colonised by *M. hominis* and was treated with moxifloxacin (moxifloxacin 400 mg p.o. for 7 days).

Table 2 shows the results of subsequent treatments where real-time PCR tested positive for *U. urealyticum* following a second antibiotic cycle administered after failure of the initial treatment. One of the 12 couples who failed treatment with azithromycin withdrew from the program. The remaining couples were treated with doxycycline 100 mg/12h p.o. for 7 days. There was only one case of therapeutic failure, which was treated alternatively with levofloxacin (500 mg/day, 7 days). As the woman remained positive, she was prescribed vaginal clindamycin 100 mg/day for 3 days. Her post-treatment test was negative.

Doxycycline proved ineffective in 4 of the 19 couples treated initially. In these cases, levofloxacin (500 mg/day, 7 days) was used as an alternative. Only one patient remained PCR-positive for *U. urealyticum*. This patient was then treated with vaginal clindamycin 100 mg/day for 3 days; she required a second course combined with a vaginal probiotic, but she still tested positive.

One of the couples who were successfully treated with levofloxacin initially for *U. urealyticum* required further treatment with moxifloxacin 400 mg p.o. for 7 days as *M. hominis* was detected in the post-treatment test (couple # 104).

The cases of *M. hominis* infection (6/7 in coinfection, 86%) were treated with azithromycin (first 2 couples with coinfection with *Ureaplasma* spp.) and doxycycline (the remaining couples). Treatments failed in 3 patients both them (of whom 2 were members of the same couple) (table 3).

The statistical analysis showed that the response to treatment was poorer among women ($p < 0.01$). In addition, azithromycin in single-dose regime was significantly less efficient as compared to doxycycline ($p < 0.01$) in both men and women.

Treatment efficacy was independent of age. However, the age range was too small to observe significant differences.

DISCUSSION

M. hominis and *Ureaplasma* spp. infections are associated with serious problems during pregnancy (chorioamnionitis, premature rupture of membranes, preterm labour, miscarriage during the last trimester of pregnancy) and/or severe infection in the newborn (bronchopulmonary dysplasia, chronic lung infection, intraventricular cerebral haemorrhage) [15, 16]. There is evidence that the use of semen infected with *Ureaplasma* spp. for *in vitro* fertilisation (IVF) reduces the rate of pregnan-

cies by embryo transfer [6] and increases no-pregnancy [5] and miscarriage rates [6, 7, 10, 14, 17]. On the other hand, the washing procedures used for semen preparation prior to IVF are not always efficient to eliminate mycoplasmas, as these may remain adhered to the surface of spermatozoa, negatively affecting their quality [13, 18, 19]. In this context, mycoplasma screening in urine samples and subsequent eradication with antibiotic treatment may provide considerable benefits in the ART protocol. Molecular techniques such as real-time PCR are highly valuable diagnostic tools as they provide significant time reductions compared to conventional cultures, are not affected by previous antibiotic use and have much higher sensitivity [20]. Our study found that 15.1% of the males were colonised by *Ureaplasma* spp. and 3.9% by *M. hominis* (practically all of whom were coinfecting with *Ureaplasma* spp.). These figures are somewhat lower than reported in earlier studies, in which colonisation by *Ureaplasma* spp. also prevailed over *M. hominis* [21–24]. Nevertheless, a high variability on genital mycoplasma prevalence has been reported in part due to geographical and methodological differences [25]. Unlike other publications which report rates of up to 5% [21, 26], there were no positive cases of *M. genitalium* in our patients. This could be explained because it's a population with a median age above 35-year-old (some studies report decreasing rates of colonisation by *M. hominis* with age [27], asymptomatic and, possibly, more conservative sexual behaviour.

Besides the benefits that the eradication of genital mycoplasma can have on the success of assisted reproduction, as already mentioned previously, it was also been shown that pre-emptive antibiotic therapy of genital mycoplasma in colonized pregnant women might represent a beneficial strategy to reduce premature labour and neonatal complications [28].

According to the recommendations of therapeutic guidelines, macrolides are the treatment of choice for *Ureaplasma* spp. infections, with doxycycline or levofloxacin as alternatives. For *M. hominis*, the treatment of choice is doxycycline, with quinolones as an alternative [29, 30]. As recommended in these guidelines and taking into account the treatment schedules used in studies in colonized women [21], azithromycin 1g/day single-dose was used to eradicate *Ureaplasma* spp. in both members of the couple when the male was positive. In addition to being a first-line option, the azithromycin regime simplifies treatment compliance. For the same reason, azithromycin was also used in cases of *Ureaplasma/M. hominis* coinfection. In several studies, azithromycin is reported to be a highly effective antibiotic against *Ureaplasma* spp., with a very low percentage of resistant strains [31–33]. However, the *in vivo* results obtained in our series using the treatment recommended for sexually transmitted infections (1 g single dose; www.vademecum.es) revealed the low efficacy of this macrolide. For this reason, after the initial results, doxycycline was used as the first choice (100 mg b.i.d. p.o., 7 days). Some reports have demonstrated that a single dose of azithromycin is less efficient than azithromycin used over longer periods [34], and this could be the reason behind its low efficacy in our series. Possibly, also could contribute its bacteriostatic mechanisms and

less efficacy at low pH. Interestingly, we have also observed a higher rate of antibiotic failure against *Ureaplasma* spp. in women, as compared to men, and this difference was statistically significant ($p < 0.01$) for azithromycin. Fluoroquinolones (levofloxacin and moxifloxacin) were used as the therapeutic alternative where doxycycline did not eradicate colonisation by genital mycoplasmas. Levofloxacin was ineffective in one couple colonised by *Ureaplasma* spp. and in another couple with *M. hominis* coinfection. Levofloxacin has been suggested as an effective antimicrobial agent against *Ureaplasma* spp. and *M. hominis* [35], but our results do not support this, as therapeutic failure occurred in 40% of patients treated with levofloxacin. Nevertheless, in view of the small number of cases, we cannot draw any firm conclusions in this regard. Moxifloxacin has been reported to be a powerful antibiotic against *M. hominis* [35] and indeed, it was a successful alternative in our series for one couple who had failed levofloxacin.

Clindamycin represent a potential well tolerated treatment for unresolved mycoplasma infections after treatment with tetracyclines and macrolides [4, 5]. In fact, this antibiotic is commonly used to treat bacterial vaginosis involving *M. hominis*. Although *Ureaplasma* spp. are considered intrinsically resistant to lincosamides, given the low percentage of sensitive strains [35, 36], we decided to use clindamycin as a third-line treatment in women showing therapeutic failure with azithromycin, doxycycline and/or levofloxacin, as it can be administered in vaginal suppositories, resulting in high local drug concentrations. Indeed, eradication of *Ureaplasma* spp. was achieved in one case. More assays are necessary to know the effect of probiotics on the success of the antibiotic treatments.

Treatment efficacy was independent of age, even though the age range was too small to observe significant differences.

Using mycoplasma-free semen we could replace intracytoplasmic microinjection by conventional "in vitro" fecundation in 7,6% of the cases (from 3% to 10,6%) which means cost savings of 7% for each case.

In summary, we consider that genital mycoplasma routine screening could be useful in order to increase the quality of semen which could simplify the *in vitro* fertilisation procedures and raise the success rate of embryo implantation. When antibiotic treatment is necessary, real-time PCR significantly reduces time to initiation of treatment and can be used to assess its efficacy. Doxycycline was more efficacious than azithromycin single-dose regime in eliminate genital mycoplasma colonization. On the other hand, eradication of genital mycoplasmas prior to gestation can avoid possible adverse effects on pregnancy, fetus or the newborn, so, according to the opinion of other authors, as genital mycoplasma colonization of cervix is strongly associated with adverse effects as placenta previa, before a planned pregnancy, eradication of this microorganisms is necessary to prevent pregnancy negative outcomes. In addition, eradication of genital mycoplasma allows, in most cases, to use conventional *in vitro* fecundation technics instead of intracytoplasmic microinjection which is more laborious, complex and expensive.

Table 3 Number of therapeutic failures depending on the antibiotic used for initial treatment in patients positive for *M. hominis*

	Men	Women	Couples
Azithromycin	0	1	1
Doxycycline	0	1	1

In the case of patients who do not eliminate colonization in the first treatment regime, the benefit of initiating successive alternative antibiotic treatments is debatable due to delayed onset of fertilization and the possible undesirable effects on the vaginal microbiota that seems to play an important role in relation to female fertility [37, 38].

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None to declare

CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest.

INFORMED CONSENT

All patients included in the study previously signed the informed consent document.

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