PŮVODNÍ PRÁCE

Prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum* in women undergoing an initial infertility evaluation

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ABSTRACT

Aims: *Mycoplasma hominis* and *Ureaplasma urealyticum* are potentially pathogenic bacterial species that are frequently isolated from the urogenital tract of women. These pathogens could be responsible for various genitourinary diseases and have been associated with adverse pregnancy outcomes and female fertility problems. The aim of this study was to analyse the presence of *M. hominis* and *U. urealyticum* in the cervical canal of uterus of women with and without fertility problems.
Methods: Endocervical swabs obtained from women with reproductive problems and fertile women were tested by both cultivation and polymerase chain reaction. The antimicrobial susceptibility to the azithromycin, ciprofloxacin, doxycycline and erythromycine of the isolated strains of *M. hominis* and *U. urealyticum* was also tested by the microdilution broth method.
Results: A total of 111 women with fertile problems were examined. *U. urealyticum* was detected in samples from 44 (39.6%) women. *M. hominis* was detected in significantly fewer samples, i.e. only from 9 (8.1%) samples. From these, 6 (5.4%) women were positive for both microorganisms. The fertile group consisted from 23 women. The presence of *U. urealyticum* was detected in 8 (34.7%) of them. *M. hominis* was detected only in the mixture with *U. urealyticum* in 3 (13.0%) cases. The most effective antibiotic against both species in our study was doxycycline.
Conclusion: The results show slightly higher incidence of *M. hominis* and *U. urealyticum* in the genitourinary tract of women with fertility problems compare with control group. The potential negative effect of these species on the reproduction ability of women was not observed.

KEYWORDS:

mycoplasma – ureaplasma – women – infertility – assisted reproduction

SOUHRN

Sleha R., Boštíková V., Hampl R., Salavec M., Halada P., Štěpán M., Novotná Š., Kukla R., Slehová E., Kacerovský M., Boštík P.: Výskyt *Mycoplasma hominis* a *Ureaplasma urealyticum* u žen s poruchou fertility

Cíle: *Mycoplasma hominis* a *Ureaplasma urealyticum* jsou podmíněně patogenní mikroorganismy, které se frekventovaně vyskytují v urogenitálním ústrojí žen. Uvedené bakteriální druhy mohou způsobovat infekce urogenitálního traktu a jsou rovněž spojovány s nepříznivým vlivem na průběh těhotenství, případně s lidskou neplodností. Cílem této práce bylo zjistit četnost výskytu *M. hominis* a *U. urealyticum* u žen z asistované reprodukce ve srovnání s kontrolní skupinou plodných žen.
Metody: Průkaz mykoplazmat v cervikálních stěrech byl prováděn kultivačním vyšetřením a polymerázovou řetězovou reakcí. U izolovaných kmenů byla následně ověřena mikrodiluční bujónovou metodou jejich citlivost k azithromycinu, ciprofloxacinu, doxycyklinu a erythromycinu.
Výsledky: Celkově bylo vyšetřeno 111 žen s poruchou reprodukční funkce. *U. urealyticum* bylo prokázáno ve stěrech 44 žen (39,6 %). Výskyt *M. hominis* byl zaznamenán pouze u 9 žen (8,1 %). Z tohoto počtu bylo 6 žen (5,4 %) pozitivních na přítomnost obou bakteriálních druhů. Kontrolní skupinu tvořilo 23 žen. Přítomnost *U. urealyticum* byla detekována u 8 žen (34,7 %). *M. hominis* bylo detekováno pouze ve směsi s *U. urealyticum*, a to ve 3 případech (13,0 %). Nejvyšší inhibiční aktivita na oba testované druhy byla pozorována u doxycyklinu.
Závěr: Výsledky ukazují na vyšší incidenci *M. hominis* a *U. urealyticum* v genitálním traktu žen s problémy v reprodukci ve srovnání s kontrolní skupinou. Potenciální negativní efekt na reprodukční schopnost žen nebyl pozorován.

KLÍČOVÁ SLOVA:

mykoplazmata – ureaplazmata – ženy – neplodnost – asistovaná reprodukce

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INTRODUCTION

Mycoplasma (*M.*) *hominis* and *Ureaplasma* (*U.*) *urealyticum* are members of unique class of microorganisms that are considered as opportunistic pathogens of humans. These microorganisms are commonly found within the genitourinary tract of healthy women, but the both species have been associated with an increased risk of developing certain pathogenic conditions, including acute urethritis, bacterial vaginosis, pelvic inflammatory disease and pyelonephritis [1–3]. In addition, numerous previous reports proposed that microbial invasion of *M. hominis* and *U. urealyticum* into the amniotic cavity were associated with adverse pregnancy outcomes. Inflammatory reactions within genitourinary tract of pregnant women represent common pathways that could lead to complicated pregnancy and initiate a premature spontaneous delivery [4–7].

Genital mycoplasmas and ureaplasmas could be transmitted from an infected genital tract of females to the fetus or neonate. Intrauterine infection caused by *M. hominis* or *U. urealyticum* can result in chorioamnionitis, dissemination to fetal organs or congenital pneumonia [8, 9]. The mycoplasma species were also isolated from the cerebrospinal fluid or from the brain of a several infants [10, 11]. There is considerable evidence for the role of *M. hominis* in post-partum and post-abortal fever [12].

Inflammation in women genital tract caused by these species could be also involved in female infertility. During past decade was observed the high frequency of *U. urealyticum* in women with fertility problems [13]. Reduced pregnancy rates after in vitro fertilisation were detected in women with cervical colonisation with ureaplasmas in comparison to women with successful embryo transfer. For women undergoing *in-vitro* fertilisation is still unclear whether or not test for these microorganisms or to prescribe antibiotics to colonised women [14]. However, the effect of the presence mycoplasmas in the genitourinary tract on the unexplained infertility remains still controversial.

The laboratory diagnosis of the *M. hominis* and *U. urealyticum* was originally based on the direct methods. The culture techniques are still valuable and sensitive, but very time-consuming. The current generation of diagnostic test is more often based on the nucleic acid amplification tests, such as polymerase chain reaction (PCR) and its modifications [15, 16, 17]. These approaches have been described as more rapid, specific and sensitive than traditionally used culture methods.

Mycoplasma or ureaplasma infections require the therapeutic use of antimicrobials. Mycoplasmas are naturally resistant to antibiotic compounds that are directed to inhibition of cell wall synthesis, such as penicillins and cephalosporins [18]. Tetracyclines, macrolides or quinolones are the major antibiotics used in the antimicrobial therapy of genital mycoplasmas [19]. However, their therapeutic efficacy may be unpredictable due to increasing resistance of these microorganisms [20].

In the present study, the prevalence of *M. hominis* and *U. urealyticum* were investigated in the two groups considered: fertile women and asymptomatic patients with fertility problems. We analysed the effect of these pathogens on the rate of conception in assisted reproduction. The antimicrobial susceptibilities of a large number of clinical isolates of *M. hominis* and *U. urealyticum* to different antibiotics were also determined.

MATERIALS AND METHODS

Study population and sample collection

Endocervical swabs and clinical data of investigated and control groups of women were collected. The study group consisted from 111 women with fertility problems, who were treated in the Centre of Assisted Reproduction in Pardubice (Czech Republic). The investigated women have not become pregnant after at least 1 year of having sex without using control methods. The fertile (control) groups were 18 female patients with pregnancy in the past, without clinical symptoms of urogenital infections from the University Hospital Hradec Kralove (Czech Republic) and 5 pregnant women in the 1st trimester of pregnancy. The patients and control groups did not receive antibiotics at least two weeks before the study. The swabs were transported to the laboratory in the mycoplasma transport medium (PPLO broth, BD, USA). The study protocol was approved by the local ethics committees of Centre Assisted Reproduction SANUS and University Hospital Hradec Kralove.

Bacterial Strains

The reference strains of *M. hominis* (CIP 103715) and *U. urealyticum* (CIP 103755) were purchased from the Collection of Institute Pasteur, Paris, France. These microorganisms were used as positive controls.

Culture media and specimen processing

M. hominis and *U. urealyticum* strains were cultivated in PPLO broth. The following composition is given for the preparation of one litre of medium: 21 g BD Difco™ PPLO broth (Becton, Dickinson and Company, USA), 200 mL horse serum (LabMediaServis, Czech Republic), 100 mL freshly-prepared yeast extract, 1 g ampicillin (Biotika Bohemia, Czech Republic), 2 mL 1% phenol red (Sigma-Aldrich, United Kingdom) and 700 mL of purified water. The culture medium contains 0.8 g thallium acetate (Sigma-Aldrich) and 5 g L-arginine for *M. hominis* (Merck, Germany) and 1 mg amphotericin B (Sigma-Aldrich), 256 mg lincomycine (Sandoz, Slovenia) and 1 mL 40% urea (Himedia, Italy) for *U. urealyticum*. PPLO agar was prepared from 35 g of BD Difco™ PPLO agar (Becton, Dickinson and Company, USA) and supplemented the same way as the PPLO broth.

Clinical samples were inoculated into the both PPLO broth medium for *M. hominis* and *U. urealyticum*. Samples were incubated under 5% of CO_2 at 37 °C and monitored daily for up to five days. A positive growth of each microorganism was considered when a colour change due to arginine or urea hydrolysis resulting in concomitant alkalization of the broth medium was observed and confirmed with the growth of typical colonies after subculture on PPLO agar. All of the specimens and clinical isolates were stored at -80 °C until DNA extraction and antimicrobial susceptibility testing were performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed in PPLO broth without inhibitors (ampicillin, amphotericin B,

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lincomycine and thallium acetate) by microdilution method. Reference standard powders of ciprofloxacin, doxycycline and erythromycin were purchased from Sigma-Aldrich (United Kingdom), while azithromycin was obtained from Teva Pharmaceuticals (Czech Republic). All tested agents were dissolved in a sterile deionized water or ethanol as directed by the manufacturer and prepared stock solution by diluting in PPLO broth. Serial 10-fold dilutions of each isolate were prepared in wells containing PPLO broth without antibiotics to determine the number of color-changing units (CCU). Each tested strain was run in duplicate on a sterile 96-well round-bottom plate composed of the 4 antimicrobials in concentration range 32–0.06 µg/mL, 1 negative control (no antimicrobials and no culture). Subsequently, each well was than inoculated of 10^4 to 10^5 CCU/mL of the standardized inoculum of tested strains and incubated under 5 per cent of CO_2 at 37 °C.

Table 1. Details of PCR primers used in this study

Target species	Primer	Sequences 5´–3´	Reference	Product size
M. hominis	MhF	CAATGGCTAATGCCGGATACGC	21.	334
	MhR	GGTACCGTCAGTCTGCAAT		
U. urealyticum	UuF	CAATCTGCTCGTGAAGTATTAC	22.	429
	UuR	ACGACGTCCATAAGCAACT		

The MIC endpoint was determined at 24–48 hours as the lowest concentration of antimicrobial agent at which the metabolism of the organism was inhibited, as evidenced by a colour change of culture medium.

Table 2. The causal factor of the infertility of women from the investigated group (n = 111) and rate of presence of genital mycoplasmas

Causality	Women with the presence of genital mycoplasmas (n = 47)	Women with the absence of genital mycoplasmas (n = 64)	p-value
Age factor	2 (4.3%)	2 (3.1%)	1.00
Andrological factor	10 (21.3%)	15 (23.4%)	0.82
Endometriosis	3 (6.4%)	3 (4.7%)	0.70
Hormonal factor	2 (4.3%)	1 (1.5%)	0.57
Immunological factor	4 (8.5%)	5 (7.8%)	1.00
Ovarian factor	13 (27.7%)	21 (32.8%)	0.68
Tubal factor	4 (8.5%)	4 (6.3%)	0.72
Unexplained	9 (19.1%)	13 (20.3%)	1.00

Legend: Categorical variables were compared using Fisher's exact test. Statistically significant results are marked in bold. Variables are presented as number (%).

DNA extraction and PCR assay

Samples were vortexed and 300 µl transferred to a QIAamp DNA Mini Kit column (Qiagen, USA). For the DNA extraction, the manufacturer's protocol was followed. The final elution volume of purified DNA was 200 µl. Isolated DNA samples were stored at -20 °C until the PCR assay performing.

The PCR was carried out in a final volume of 25 µL reaction mixture with 10x PCR-buffer (100 mM Tris-HCl, pH 8.3; 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, 0.5 µM of each oligonucleotide, 1.25 U *ExTaq* polymerase (Takara Bio Inc., Shiga, Japan), and 2 µl PCR templates (added last). Purified mycoplasmal DNA was used as a positive control for amplification. The published primer sequences targeted the highly conserved region of the 16S rDNA gene of *M. hominis* and amplified a unique 334 bp region [21]. *U. urealyticum* primers were obtained from the urease gene sequence (Table 1), a 429-bp fragment of the gene was amplified with this primer set [22]. The amplification was performed in a TP Profesional cycler (Biometra, Germany). The cycling conditions to amplify consisted of an initial denaturation at 94 °C for 4 min, 30 cycles of melting at 94 °C for 1 min, annealing at 62 °C for 1 min, and elongation at 72 °C for 1 min and a final extension at 72 °C for 1 min fragment analyzed by gel electrophoresis on 1.3% (w/v) agarose gels and visualised after staining with ethidium bromide using a UV transilluminator (Vilber Lourmat, France). Negative and positive controls were used in each experiment run.

RESULTS

During the study period June 2011 and March 2014 a total of 111 women with fertile problems and 23 women from control groups were investigated. The mean age for the study group was 34.3 ± 3.9 (ranged 24–47) years and 33.7 ± 5.4 (ranged 23–44) years for the control cohort. The causal factors of the infertility of women from the investigated group are presented in the Table 2.

Of the 111 subjects from the investigated group, 47 (42.3%) were by culture and PCR positive for *M. hominis* and/or *U. urealyticum*. Of these, 3 (2.7%) patients were positive only for *M. hominis* and 38 (34.2%) for *U. urealyticum*. In 6 (5.4%) cases were detected both species (Table 3). Among 23 fertile women from the control cohort were 8 (34.7%) positive for the presence of *M. hominis* and/or *U. urealyticum*. *M. hominis* has been found only in the mixture with *U. urealyticum* in 3 (13.0%) cases. *U. urealyticum* alone was detected in 5 (21.7%) samples (see Table 3). The presence of *M. hominis* and *U. urealyticum* in the relation with the causal factor of infertility is presented in the Table 2. In this study, the presence of the genital mycoplasmas was not associated with the higher rates of incidence for some causal factor of infertility.

The association between the presence of mycoplasmas and rates of conception in the cohort of infertile women was also monitored. Results for a total of 109 patients were obtained from the clinic of assisted reproduction (Table 4). No significant differences in the rate of conception between the group of pregnant and non-pregnant women from the assisted reproduction were observed.

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Table 3. Presence of *U. urealyticum* and *M. hominis* in the cervical swabs from fertile and infertile women

Microorganisms	Infertile women (n = 111)	Fertile women (n = 23)	p-value
M. hominis	3 (2.7)	0 (0.0)	1.00
U. urealyticum	38 (34.2)	5 (21.7)	0.33
M. hominis + U. urealyticum	6 (5.4)	3 (13.0)	0.18
Total	47 (42.3)	8 (34,7)	0.64

Legend: Categorical variables were compared using Fisher's exact test. Statistically significant results are marked in bold. Variables are presented as number (%).

Table 4. Presence of *M. hominis* and *U. urealyticum* and the rate of conception in infertile women from the clinic of assisted reproduction

MH and/ or UU	Non-pregnancy		Pregnancy	
	N. of patients	Percentage	N. of patients	Percentage
Positive	41	41.4	4	40.0
Negative	58	58.6	6	60.0

The minimal concentrations at which 50% (MIC50) and 90% (MIC90) of isolated strains were inhibited are shown in Table 5. Doxycycline was the most effective antibiotics against both tested species with MIC90 0.125 μg/mL for *M. hominis* and 0.5 μg/mL for *U. urealyticum*. In the present study, erythromycin and azithromycin susceptibility testing of *M. hominis* clinical isolates using microdilution method demonstrated a high frequency (100%) of resistance. The value of MIC90 of azithromycin and erythromycin for *U. urealyticum* were 4 and 8 μg/mL respectively. Ciprofloxacin inhibited growth of the *M. hominis* strains with MIC90 2 μg/mL and 16 μg/mL for *U. urealyticum*.

DISCUSSION

The presence of pathogenic microorganisms in the genitourinary tract of women could represent significant aetiologic factor that affects the female reproduction ability. *M. hominis* and *U. urealyticum* are common inhabitants in the cervices of many sexually active women and are associated with several urogenital infections (bacterial vaginosis, cervicitis, pelvic inflammatory disease). The inapparent colonisation by mycoplasmas or ureaplasmas could induce pro-inflammatory immune response in the endometrium that may an adverse effect on outcome of pregnancy [1, 3, 4]. However the pathological role of these species in the aetiology of the female fertility problems is still unknown.

To our best knowledge, this is the first report on the prevalence of *M. hominis* and *U. urealyticum* in the genitourinary tract of women from the assisted reproduction in the Czech Republic. In our study, the high prevalence of *M. hominis* and *U. urealyticum* (42.3%) was detected in the investigated group of infertile women in comparison with control subjects (34.7%). The frequency of the *U. urealyticum* was higher than that of *M. hominis* in both groups of women. These findings are consistent with other studies. The study between infertile and fertile couples showed significantly higher presence of these species in couples with fertility problems [23]. The similar prevalence of both microorganisms as in this study between women with fertility problems was obtained by Zdrodowska et al. (2006), who reveal the genital carriage of *M. hominis* and *U. urealyticum* in 1 (2.3%) and 16 (37.2%) cases respectively [24]. In contrast, another study detected no difference among fertile and infertile groups with respect of prevalence of *M. hominis* and *U. urealyticum* [25]. In the similar study, Witkin et al. (1995) detected significant lower colonisation rate of both species in cervices of women undergoing in vitro fertilisation, compared with our results. According to authors, *U. urealyticum* is present in the cervices of many culture-negative women. Its presence, however, does not influence IVF outcome subsequent to embryo transfer in women treated with tetracycline after oocyte retrieval [26]. In our study no significant differences in the rate of conception in relation to the presence of investigated species were observed. However to confirmation these results is to necessary monitoring in the greater number of patients.

The antimicrobial susceptibility testing of isolated strains was also performed. It is necessary to monitor the local drug situation to provide reasonable information for clinics. Doxycycline was the most effective antimicrobials against all of tested isolated strains of *M. hominis* and *U. urealyticum* in this study. These results confirmed that doxycycline could be still the drug of first choice for the treatment of the urogenital infections caused by mycoplasmas or ureaplasmas. However, local antimicrobial susceptibility testing is recommended due to

Table 5. Antimicrobial susceptibility of clinical isolates of *M. hominis* and *U. urealyticum* from women with fertile problems

Antimicrobial	U. urealyticum MIC (μg/mL)			Antimicrobial	M. hominis MIC (μg/mL)		
	Range	MIC50	MIC90		Range	MIC50	MIC90
Azithromycin (n = 27)	0,06–16	4	4	Azithromycin (n = 5)	> 32	> 32	> 32
Ciprofloxacin (n = 32)	1 ≥ 32	4	16	Ciprofloxacin (n = 5)	0,5–2	1	2
Doxycycline (n = 33)	0,25–0,5	0,5	0,5	Doxycycline (n = 5)	0,125	0,125	0,125
Erythromycine (n = 32)	0,125-> 32	2	8	Erythromycine (n = 5)	> 32	> 32	> 32

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high rate of mycoplasma resistance against tetracyclines. Tetracycline resistance is associated with the presence of transposon-borne tetM determinant [27]. The prevalence of *tetM* gene between clinical isolates of *M. hominis* is between 17-30% [28, 29]. The resistance to tetracyclines was described also for Ureaplasma ssp. isolates [30]. The high *in-vitro* activity was determined for ciprofloxacin which can be used as an alternative for the tetracyclines. But in recent years, high-level resistance to various fluoroquinolones of *M. hominis* strains were reported [31, 32]. Similar results for doxycycline and ciprofloxacin have been reported from Krausse et al. (2010) [31]. All of the tested strains of *M. hominis* were resistant against azithromycin and erythromycin. *M. hominis* is generally resistant to 14- and 15-membered macrolides. This resistance has been mainly associated with a G2057A transition in domain V of 23S rRNA [33]. The lower inhibitory effect of erythromycin against clinical isolates of *U. urealyticum* was also recently reported [20].

CONCLUSION

Results of this work show higher cervical colonisation of *M. hominis* and *U. urealyticum* in infertile women compared with fertile group. However, in the present study the rate of conception in assisted reproduction treatment was not significantly affected by the presence of investigated microorganisms. Thus, the potential negative effect of both bacteria on the reproduction ability of women remains controversial. But, this colonisation may be predictive factor of prenatal and perinatal complications. Our results also indicate that doxycycline and ciprofloxacin are effective drugs against both microorganisms when empirical therapy is required.

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