

# BMJ Open Unusually low prevalence of *Mycoplasma genitalium* in urine samples from infertile men and healthy controls: a prevalence study

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## ABSTRACT

**Objective:** To detect *Mycoplasma genitalium* in urine samples of infertile men and men without any signs of infection in order to investigate whether *M. genitalium* and other genital mycoplasmas (*Mycoplasma hominis* and *Ureaplasma* spp) are found more often in urine samples of infertile men than in asymptomatic controls and to determine resistance to macrolides.

**Methods:** The study included first void urine samples taken from 145 infertile men and 49 men with no symptoms of urethritis. *M. genitalium*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were detected by commercial PCR. *Trichomonas vaginalis* was detected by microscopy and culture. *M. hominis* and *Ureaplasma* spp were detected by culture. *M. genitalium* was detected by in-house conventional and real-time PCR.

**Results:** Two *M. genitalium* positive samples were found among samples obtained from infertile men. All asymptomatic men were *M. genitalium* negative. Macrolide resistance was not found in either of the two positive samples.

**Conclusions:** In comparison with reported data, an unusually low prevalence of *M. genitalium* was found in infertile men. The reasons for this unexpected result are not known; possibly, local demographic and social characteristics of the population influenced the result. Further studies to investigate *M. genitalium* in infertile and other groups of patients are needed.

## INTRODUCTION

Reliable detection of *Mycoplasma genitalium* became possible after the development of PCR assays.<sup>1–2</sup> The prevalence of *M. genitalium* in patients with non-gonococcal urethritis (NGU) ranges from 13% to 42%; and in asymptomatic men from 0% to 15%.<sup>2–4</sup> The impact of *M. genitalium* on male fertility remains unclear.<sup>2–5</sup>

Our aim was to detect *M. genitalium*, *M. hominis* and *Ureaplasma* spp in first void urine (FVU) samples of infertile men and men

## Strengths and limitations of this study

- We are aware of low number of urine samples in both groups of participants.
- These are only preliminary results, and were unexpected in comparison with reported data. However, the results were confirmed in two independent laboratories to exclude the possibility of laboratory error.
- We plan to continue this study, collecting urine samples from a larger number of infertile men and comparing them both with men with urethritis and asymptomatic men.
- If the results are similar (ie, an unexpectedly small number of men with *Mycoplasma genitalium*), we will try to determine the reason for this.

without any symptoms and/or signs of infection and, additionally, to determine the prevalence of macrolide resistance in *M. genitalium*. We restricted our study to infertile men without the following common sexually transmitted infections (STIs): *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae*.

To our knowledge, this is the first study in Croatia which has been undertaken to detect *M. genitalium*.

## METHODOLOGY

The study was approved by the ethics committee of the School of Medicine, University of Zagreb. It is part of the Croatian Ministry of Science grant (108-1080114-0014): “Molecular detection of microorganisms: their influence on antimicrobial consumption”.

Each participant provided written informed consent, and completed a questionnaire stating reasons for attendance, age, symptoms of urethritis, number of lifetime

sexual partners, history of STIs and recent/current antibiotic treatment.

FVU (about 20 mL) was taken from the patients; 4–5 mL of each sample was used for culture of *M. hominis*, ureaplasmas and *T. vaginalis*. For PCR detection of *M. genitalium*, *C. trachomatis* and *N. gonorrhoeae*, 4–5 mL of each sample were used. Five millilitres of the original FVU were frozen and shipped to Staten Serum Institut in Copenhagen for confirmation by real-time *M. genitalium* PCR. Samples were immediately processed for *M. hominis*, *T. vaginalis* and ureaplasmas. For PCR detection samples were stored at  $-20^{\circ}\text{C}$ .

*C. trachomatis* and *N. gonorrhoeae* were detected by PCR (Cobas TaqMan CT/NG Test, V.2.0 Roche Diagnostic, Basel, Switzerland), as described by the manufacturer, and urethral swabs were obtained for detection of *N. gonorrhoeae* by culture (BBL MTM, New Jersey, USA). *T. vaginalis* was detected by microscopy and culture in modified Diamonds medium (Remel, Inc, Santa Fe, USA). Thirteen of the infertile men and three controls were excluded owing to infection with a recognised STI (*Chlamydia trachomatis*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae*), leaving a total of 194 FVU samples. These were collected in polypropylene containers (Sarstedt, Nümbrecht, Germany) from men who were referred to the Department of Clinical Microbiology and Department of Dermatology, Clinical Hospital Centre Zagreb. One hundred and forty-five samples were obtained from men as a part of an infertility investigation. None of them had any symptoms of genitourinary infections, but all had an abnormal semen quality analysis (oligozoospermia, asthenozoospermia, oligoasthenozoospermia). Infertility of their female partners was excluded by hysterosonosalpingography.

Forty-nine samples were from asymptomatic men attending the clinic as a part of an annual physical examination.

Aliquots of the urine samples (4–5 mL) were prepared for culture of genital mycoplasmas and for molecular testing, respectively. Urine samples were centrifuged at  $3000\times g$  for 5 min and sediments were inoculated in urea-arginine broth (bioMerieux, Lyon, France) and onto A7 agar (Becton Dickinson, Cockeysville, Maryland, USA). The vials were incubated for 48 h at  $37^{\circ}\text{C}$ . The agar plates were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 5 days and examined microscopically for the appearance of typical mycoplasma colonies.<sup>6</sup>

The rest of the urine (4–5 mL) was concentrated at  $20000\times g$  for 15 min at  $4^{\circ}\text{C}$ . The pellet was resuspended in 200  $\mu\text{L}$  of 20% w/v Chelex 100 slurry (Sigma, USA) in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The mixture was vortexed for 1 min; then placed in a thermoblock for 10 min at  $95^{\circ}\text{C}$ . The mixture was centrifuged briefly and the supernatant aspirated into new tubes and stored at  $-20^{\circ}\text{C}$  until required for PCR.<sup>7</sup>

PCR for detection of *M. genitalium* was performed with two pairs of primers: the first targeted the 16S rRNA gene: 16SFG2 (5'-CCT TAT CGT TAG TTA CAT

TGT TTA A), 16SRG (5'-TGA CAT GCG CTT CCA ATA AA),<sup>7</sup> and the second targeted the MgPa major adhesin gene: MgPa1 (5'-AGT TGA TGA AAC CTT AAC CCC TTG G), MgPa3 (5'-CCG TTG AGG GGT TTT CCA TTT TTG C).<sup>1</sup>

All samples were examined by both assays with internal controls for PCR inhibition, and both PCRs were performed as previously described.<sup>1 7–9</sup>

The PCR was performed in an automated DNA thermal cycler (PCR System 9700, Applied Biosystems).

To confirm the results, an aliquot of the original FVU samples (5 mL) was shipped to Statens Serum Institut, Copenhagen, Denmark, where it was tested by an inhibitor-controlled real-time PCR using primers detecting the MgPa gene, as previously described.<sup>7 9</sup>

Macrolide resistance mediating mutations in region V of the 23S rRNA gene was detected by DNA sequencing of amplicons obtained directly from the clinical specimens, and performed at Statens Serum Institute Copenhagen, Denmark.<sup>10</sup>

STATISTICA (data analysis software system), V.10 (StatSoft, Inc (2011), USA) was used for data analysis. The median was used to describe the age of groups and the number of observations and percentage to describe categorical variables. To compare age between the groups a Mann–Whitney U test was used, and to compare categorical variables a  $\chi^2$  test was used. A p value of  $<0.05$  was considered to be statistically significant for all tests performed.

## RESULTS

The infertile men were comparable to the controls for age ( $z=-0.805$ ,  $p=0.421$ , Mann–Whitney U test). They significantly more often reported a history of STIs ( $\chi^2=14.443$ ,  $df=1$ ,  $p=0.0001$ ) and a higher number of lifetime sexual partners ( $\chi^2=35.734$ ,  $df=2$ ,  $p<0.0001$ ; table 1).

Thirteen of the infertile men and three controls were excluded owing to infection with a recognised STI (*Chlamydia trachomatis*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae*).

**Table 1** Demographic and epidemiological data for all groups

Data	Infertile men (N=145)	Asymptomatic men (N=49)
Age, median (years)	38	41
History of STIs*	81 (55.8)	12 (24.4)
No of lifetime partners†		
<5	15 (10.3)	13 (26.5)
5–10	123 (84.8)	25 (51.0)
>10	7 (4.8)	11 (22.4)

Results are shown as number (%) unless stated otherwise.

\* $p=0.0001$ ,  $\chi^2$  test;

† $p<0.0001$ ,  $\chi^2$  test.

STIs, sexually transmitted infections.

Among the infertile men one patient had *N. gonorrhoeae* infection, one patient was diagnosed with *T. vaginalis* and 11 patients were *C. trachomatis* positive. These samples were also tested for *M. genitalium*, but all were negative.

*Ureaplasma* spp and *M. hominis* were isolated from the same proportion of infertile men and asymptomatic controls ( $\chi^2=0.435$ ,  $p=0.509$ ;  $\chi^2=0.021$ ,  $p=0.886$ ), respectively. Only two samples were positive for *M. genitalium*; both in the group of infertile men (2/145; 1.4%; 95% CI 0.2% to 4.9%). These men were aged 29 and 37 years, respectively, and reported three and seven lifetime sexual partners compared with the majority of the group who had had 5–10 partners.

In our laboratory we used conventional in-house PCR (qualitative) and results were confirmed at the Staten Serum Institut in Copenhagen, Denmark by real-time PCR. All *M. genitalium* results were concordant when the samples were examined by real-time PCR in Copenhagen. *M. genitalium* load for two positive samples was 778 copies/mL (c/mL) and 6765 c/mL, respectively. The man with a *M. genitalium* load of 778 c/mL was diagnosed with oligozoospermia, and the other (*M. genitalium* load of 6765 c/mL) with asthenozoospermia.

*Ureaplasma* spp were found in 30% (43/145) of the infertile men compared with 35% (17/49) of the asymptomatic men. *M. hominis* was positive in 21% (31/145) and 20% (10/49) of infertile men and asymptomatic men, respectively.

In 12 samples from the infertile men co-infection with *Ureaplasma* spp. and *M. hominis* was found. In the group of asymptomatic men co-infection in three samples was found. In the two samples with positive *M. genitalium*, taken from infertile men, no other pathogens were present.

Macrolide resistance mediating mutations in the 23S rRNA gene of *M. genitalium* were not found in either of the two positive samples.

## DISCUSSION

This study demonstrates a low prevalence of *M. genitalium* in infertile men in the Zagreb region, Croatia. Ureaplasmas and *M. hominis* were often detected in both infertile men and healthy controls, suggesting that they should be considered commensals.

FVU samples were used because several studies have reported that molecular methods performed on urine samples can detect as many, or even more, infected patients than traditional urethral swabs, or cervical swabs or semen.<sup>5–7</sup> No data for the prevalence of genital mycoplasmas in Croatia exist.

In this study the prevalence of *Ureaplasma* spp did not differ significantly among infertile men and asymptomatic controls, and was present in about one-third of both groups. This strongly suggests that ureaplasmas do not have a significant role of in male infertility.

We did not perform a specific test for the *Ureaplasma* spp. Most of the published studies have reported the

prevalence of ureaplasmas in infertile men without discriminating between *U. urealyticum* and *U. parvum*. The data are not conclusive about the prevalence of *U. urealyticum* and *U. parvum*. Abusaraha *et al*,<sup>11</sup> found that *U. parvum* was the most prevalent isolate detected among infertile men (90%).

*M. hominis* was detected in 20% of asymptomatic men and 21% of infertile men, respectively, a higher prevalence than in some other studies.<sup>12</sup> *M. hominis* is considered normal flora of the urethra and the prevalence of *M. hominis* may reflect a high prevalence of bacterial vaginosis in the men's sexual partners, as *M. hominis* is known to be strongly associated with this condition in women.<sup>13</sup>

The prevalence of *M. genitalium* varies significantly in different populations<sup>2</sup> and was low in our study. Other studies have also found that *M. genitalium* is uncommon in the FVU of infertile men.<sup>5</sup> The two positive samples in our study were from the group of 145 infertile men and *M. genitalium* was not detected in any of the controls. We were concerned that technical problems might have caused the low prevalence, and therefore, frozen FVU samples were shipped to Copenhagen for evaluation. However, a 100% concordance between the results was found, suggesting that the prevalence of *M. genitalium* in this Croatian population is, indeed, very low.

A possible relationship between infection and infertility has been the subject of controversy for years. It is estimated that only 15% of male infertility is related to genital tract infection.<sup>5–14</sup> Detection of bacteria in urogenital samples does not necessarily suggest infection but may signify colonisation, contamination or infection.

Only a few studies have examined the association between *M. genitalium* and male infertility<sup>5–15</sup> and these studies did not have control populations of fertile men. We tried to design a study in which all other potential infective causes of infertility were excluded. In recent studies the prevalence of *M. genitalium* in infertile men was similar to the prevalence found by us (1.4%): 4.8% in the study of Gdoura *et al*<sup>5</sup> and 3.2% was in the study of Abusaraha *et al*.<sup>11</sup>

Findings for *C. trachomatis*, which is the most common bacterial cause of NGU, were similar. *C. trachomatis* in women is a well-established cause of tubal factor infertility; in men it causes NGU. Also, it has been shown that *C. trachomatis* attaches to spermatozoa (on the surface and in the nucleolus). However, its role in male infertility, like the role of *M. genitalium*, is not yet clear. There are significant variations in the prevalence of *C. trachomatis* infections in men with infertility ranging from 0% to 42.3%, depending on the methodology, type of sample and differences of infection rates in different populations. In a recently published Canadian study<sup>16</sup> the prevalence of *C. trachomatis* infection in 5588 infertile men, was 0.3%. The author concluded that this low prevalence clearly demonstrates that a small proportion of male infertility is caused by *C. trachomatis*.<sup>17</sup>

We attempted to study an asymptomatic group of men without urethritis as controls, and found that all were

negative for *M. genitalium*. Unfortunately, the infertile men had had significantly more partners and also reported previous STIs more commonly than did the controls, suggesting that the control group had less risky behaviour.

Both *M. genitalium* positive samples were tested for macrolide resistance and were susceptible. This is encouraging considering the widespread use of azithromycin in the treatment of chlamydia and unspecific urethritis in Croatia. It is not possible to provide estimates of the prevalence of resistance to macrolides in this bacterium. Obviously, more *M. genitalium* positive samples should be tested in order to guide future treatment guidelines.

An unusually low percentage of *M. genitalium* was found in this study. The reasons for this unexpected result cannot be explained. Further studies to investigate *M. genitalium* in infertile and other group of patients from Croatia are needed.

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**Contributors** VP designed the study, was responsible for questionnaire data and data analysis, and wrote part of the manuscript; LZ-S tested samples for *Mycoplasma genitalium*, and drafted the article; VT planned the study and wrote part of the text; MS collected samples and edited the manuscript; SL collected samples and questionnaire data; JSJ tested samples for *M. genitalium*, edited the manuscript and approved the final version; SP tested samples and corrected the draft version; IM revised the manuscript.

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**Patient consent** Obtained.

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