BMJ Open

Unusually low prevalence of Mycoplasma genitalium in urine samples from infertile men and healthy controls

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2014-005372
Article Type:	Research
Date Submitted by the Author:	31-Mar-2014
Complete List of Authors:	Plecko, Vanda; Clinical Hospital Center Zagreb, Department for Clinical and Molecular Microbiology; University of Zagreb, School of Medicine Zele-Starcevic, Lidija; Clinical Hospital Center Zagreb, Department for Clinical and Molecular Microbiology Tripkovic, Vesna; Clinical Hospital Center Zagreb, for Clinical and Molecular Microbiology Skerlev, Mihael; Clinical Hospital Center Zagreb, Department of Dermatology and Venereology ; University of Zagreb, School of Medicine Ljubojevic-Hadzavdic, Suzana; Clinical Hospital Center Zagreb, Department of Dermatology and Venereology ; University of Zagreb, School of Medicine Plesko, Sanja; Clinical Hospital Center Zagreb, Department for Clinical and Molecular Microbiology Jensen, Jørgen; Statens Serum Institut, Mycoplasma Laboratory
Primary Subject Heading:	Sexual health
Secondary Subject Heading:	Epidemiology, Infectious diseases, Sexual health
Keywords:	MICROBIOLOGY, EPIDEMIOLOGY, Molecular diagnostics < INFECTIOUS DISEASES

SCHOLARONE™
Manuscripts

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Unusually low prevalence of *Mycoplasma genitalium* in urine samples from infertile men and healthy controls.

Vanda PLECKO[1], Lidija ZELE-STARCEVIC[2], Vesna TRIPKOVIC[2], Mihael SKERLEV[3], Suzana LJUBOJEVIC[3], Sanja PLESKO[2], Jorgen Skov JENSEN[4]

[1] Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb and School of Medicine University of Zagreb, Zagreb Croatia

[2] Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb Croatia

[3] Department of Dermatology and Venerology, Clinical Hospital Centre Zagreb and School of Medicine University of Zagreb, Zagreb Croatia

[4] Mycoplasma Laboratory, Staten's Serum Institute, Copenhagen, Denmark

Corresponding author: Vesna TRIPKOVIC M.D., Ph.D, Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb Croatia. Tel: + 385 1 2367 318; fax: + 385 1 2367 393; e-mail: vbtripkovic@gmail.com

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

ABSTRACT

Objective: The goal of this study was to detect *M. genitalium* in urine samples of infertile men and men without any signs of infection. We wanted to investigate whether *M. genitalium* and other genital mycoplasmas (*M. hominis* and *Ureaplasma* sp.) are found more often in urine samples of infertile men then in asymptomatic controls as well as to determine resistance to macrolides.

Methods: The study included first void urine samples (FVU) taken from 145 infertile and 49 men without symptoms of urethritis. *M. genitalium. C. trachomatis* and *N. gonorrhoeae* were detected by commercial PCR. *T. vaginalis* was detected by microscopy and culture. *M. hominis* and *Ureaplasma* sp. were detected by culture. *M. genitalium* were detected by *in house* PCR, conventional and real-time.

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

Conclusion: Comparing to literature data, unusually low prevalence of *M. genitalium* in infertile men was found. The reasons for such unexpected result could not be documented at this moment; probably local demographic and social characteristics of population could influence the result. So, further studies designed to investigate *M. genitalium* in infertile and other group of patient populations are needed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Strenghts and limitations of this study:

- We are aware of low number of urine samples in both groupes of participants.
- These are only preliminary results, which were unexpected regarding the literature data. These results were confirmed in two independent laboratories, to exclude laboratory error.
- We are planning to continue this study to collect urine samples from larger number of infertile men and compare them with men with urethritis and asymptomatic men as well.
- If the results will be similar (unexpected low number of *M. gentialium*), we will try to determine the reason for such unusually low number of M.g. in our population.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

INTRODUCTION

Reliable detection of *Mycoplasma genitalium* became possible after the development of PCR assays (1,2). The prevalence of *M. genitalium* in patients with non-gonococcal urethritis (NGU) ranges from 13%-42%; and in asymptomatic men from 0% to 15%, respectively. (2,3,4). The impact of *M. genitalium* on male fertility remains unclear (2,5).

Our aim was to detect *M. genitalium, M. hominis* and *Ureaplasma spp.* in FVU samples of infertile and in men without any symptoms and/or sign of infection. Furthermore, we intended to determine the prevalence of macrolide resistance of *M. genitalium*. We restricted our study to infertile men in whom common STIs (*Chlamydia trachomatis, Trichomonas vaginalis, and Neisseria gonorrhoeae)* were excluded**.**

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

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

METHODOLOGY

The study was approved by the Ethics Committee, 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 filled in a questionnaire regarding reasons of attendance, age, symptoms of urethritis, number of lifetime sexual partners, history of STIs, and recent/current antibiotic treatment.

C. trachomatis and *N. gonorrhoeae* were detected by PCR (Cobas TaqMan CT/NG Test, v2.0 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). Among the infertile men, 13 were excluded due to infection with a recognised STI (CT, TV, NG), and 3 among the controls, respectively. After the exclusion, a total of 194 FVU samples remained. They were collected in polypropylene containers (Sarstedt, Nümbrecht, Germany) from men who were referred to the Department of Clinical Microbiology and Department of Dermatology, CHC Zagreb. 145 samples were from men as a part of work-up for infertility investigation. None of them had any symptom of genitourinary infections, but all of them had reports of different degree of abnormal semen quality analysis (oligozoospermia, asthenozoospermia, oligoasthenozoospermia). In their female partners tubal factors of infertility were excluded. 49 samples were from asymptomatic men attending the clinic as a part of an annual physical examination.

Aliquots of the urine samples were prepared for culture of genital mycoplasmas and molecular testing, respectively. Urine samples were centrifuged at 3000x *g* for 5 min and sediments were inoculated in urea-arginin broth (bioMerieux, Lyon, France) and onto A7 agar

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

(Becton Dickinson, Cockeysville, MD, USA). The vials were incubated 48 hours at 37°C. The agar plates were incubated at 37°C in 5% CO_2 for 5 days and examined microscopically for the appearance of typical mycoplasma colonies (6).

The rest of urine was concentrated at 20000 x *g* for 15 minutes at +4°C. The pellet was resuspended in 200 µL 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 minute; then placed in a thermoblock for 10 minutes at 95°C. The mixture was centrifuged shortly and the supernatant was aspirated in new tubes and stored at -20°C until PCR was performed (7).

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) (7), 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) (1).

All samples were performed with both assays with internal controls for PCR inhibition and both PCRs were performed as described previously (1,7,8,9). To confirm the results, an aliquot of the original FVU samples 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 (7,9).

Macrolide resistance mediating mutations in region V of the 23S rRNA-gene were detected by DNA sequencing of amplicons obtained directly from the clinical specimens, and performed at Statens Serum Institute Copenhagen, Denmark (10).

STATISTICA (data analysis software system), version 10 (StatSoft, Inc. (2011), USA) was used for data analysis. Median was used to describe age of groups and number of observations and percentage to describe categorical variables. To compare age between groups Mann-

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Whitney U test was used, and to compare categorical variables chi-square test was used. P of <0.05 was considered as statistically significant for all tests performed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

RESULTS

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

Table 1. Demographic and epidemiological data for all groups

	Infertile men N=145	Asymptomatic men N=49
Median age (years)	38	41
History of STIs[a]	81 (55.8%)	12 (24.4%)
No of lifetime partners[b]		
<5	15 (10.3%)	13 (26.5%)
5-10	123 (84.8%)	25 (51.0%)
>10	7 (4.8%)	11 (22.4%)

[a] p=0.0001, Chi-square test

[b] p<0.0001, Chi-square test

Among the infertile men, 13 were excluded due to infection with a recognised STI (CT, TV, NG), and 3 among the controls, respectively.

Ureaplasma spp. and *M. hominis* were isolated from the same proportion of infertile men and asymptomatic controls (chi-square=0.435, p=0.509; chi-square=0.021, p=0.886), respectively. Only two samples were positive for *M. genitalium*; both in the group of infertile men (2/145;

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

1.4%; 95% CI 0.2-4.9%). These men were 29 and 37 years-of-age, respectively and reported 3 and 7 lifetime sexual partners compared to the majority of the group having 5-10 partners.

All *M. genitalium* results were concordant when the samples were examined by real-time PCR in Copenhagen.

Ureaplasma spp. was positive in 30% of the infertile men compared to 35% of the asymptomatic men. *M. hominis* was positive in 21% and 20% of infertile men and asymptomatic men, respectively.

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

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

DISCUSSION

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

First void urine samples were used because several studies have reported that molecular methods performed on urine samples are able to detect as many or even more infected patients than traditional urethral swabs, or cervical swabs or semen (5,7). 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, being present in approximately one third in both groups. This strongly argues against a significant role of ureaplasmas in male infertility.

M. hominis was detected in 20% of asymptomatic men and 21% of infertile men, respectively, a higher prevalence than in some other studies (11). *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 sexual partners of the men, as *M. hominis* is known to be strongly associated with this condition in women (12).

The prevalence of *M. genitalium* varies significantly in different populations (2) but was found in at a very low prevalence in the present study. Other studies have also found *M. genitalium* uncommonly in FVU of infertile men (5). The two positive samples were from the group of 145 infertile men and *M. genitalium* was not detected in any of the controls. We were concerned that technical issues were the reason for the low prevalence, and therefore, frozen FVU samples were shipped to Copenhagen for evaluation. However, a 100%

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

concordance between the results was found, suggesting that the prevalence of *M. genitalium* in this Croatian population is, indeed, very low.

The 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 (5,13). Detection of bacteria in urogenital samples does not necessarily suggest infection because detection of bacteria may signify colonization, contamination or infection. Only a few studies have examined the association between *M. genitalium* and male infertility (5,14) and these studies suffer from any control populations of fertile men. We attempted to study an asymptomatic group without urethritis as controls, and found all men negative for *M. genitalium*. Unfortunately, the infertile men had significantly more partners and also reported previous STIs more commonly than did the controls suggesting that the control group had less risk-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**.**

Unusually low percent of *M. genitalium* was found in this study. The reasons for unexpected result could not be explained yet. Further studies designed to investigate *M. genitalium* in infertile and other group of patients from Croatia are needed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

ACKNOWLEDGEMENTS

We wish to thank Birthe Dohn for excellent technical assistance with *M. genitalium* testing at Statens Serum Institut, Copenhagen, Denmark.

Funding: This work was supported by theCroatian Ministry of Science grant No 108-1080114-0014

Contributorship Statement: V.P. has designed the study, was responsible for questionnaire data and data analysis, and wrote the part of the manuscript; L.Z.S. was responsible for testing samples for M.g, as well as in drafting of the article; V.T. participated in planning the study and wrote part of the text; M.S. participated in collecting samples and edited the manuscript; S.Lj. participated in collecting samples and questionnaire data; JSJ participated in testing samples for M.g. and approved the final version and edited the manuscript; S.P. participated in testing samples and corrected the draft version.

Conflict of interest

None declared.

Data sharing statement:

No additional data available.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

REFERENCES

1. Jensen JS, Uldum SA, Sondergard-Andersen J, et al. Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J Clin Microbiol* 1991; 29:46-50.
2. Taylor-Robinson D, Jensen JS. *Mycoplasma genitalium*: from Chrysalis to multicolored butterfly. *Clin Microbiol Rev* 2011; 24(3):498-514. doi: 10.1128/CMR.00006-11. Review
3. Ross JDC, Jensen JS. *Mycoplasma genitalium* as a sexually transmitted infection: implications for screening, testing, and treatment. *Sex Transm Infect* 2006; 82; 269-71.
4. Ishihara S, Yasuda M, Ito S, *et al*: *Mycoplasma genitalium* urethritis in men. *Int J Antimicrobial Agents* 2004; 24S:*S23-S27.*
5. Gdoura R, Kchaou W, Ammar-Keskes L, *et al*. Assessment of *Chlamydia trachomatis*, *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis*, and *Mycoplasma genitalium* in semen and first void urine specimens of asymptomatic male partners of infertile couples. *J Androl* 2008; 29(2):198-206. Epub 2007 Dec 12
6. Shepard MC, Lunceford CD. Differential agar medium (A7) for identification of *Ureaplasma urealyticum* (human T mycoplasmas) in primary cultures of clinical material. *J Clin Microbiol* 1976;3:613-625.
7. Jensen JS, Björnelius E, Dohn B, *et al*. Comparison of first void urogenital swab specimens for detection of *Mycoplasma genitalium* and *Chlamydia trachomatis* by polymerase chain reaction in patients attending a sexually transmitted disease clinic. *Sex Transm Dis* 2004; 31(4):499-507.

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

8. Eastick K, Leeming JP, Caul EO, *et al*. A novel polymerase chain reaction assay to detect *Mycoplasma genitalium*. *Mol Pathol* 2003; 56(1):25-8.

9. Jensen JS, Björnelius E, Dohn B, *et al*. Use of TaqMan 5' nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *J Clin Microbiol* 2004;42:683-692.

10. Jensen JS. Protocol for the Detection of *Mycoplasma genitalium* by PCR from Clinical Specimens and Subsequent Detection of Macrolide Resistance-Mediating Mutations in Region V of the 23S rRNA Gene. *Methods in Molecular Biology* 2012;903: 129-139.

11. Salmeri M, Valenti D, La Vignera S, et al. Prevalence of *Ureaplasma urealyticum* and *Mycoplasma hominis* infection in unselected infertile men. *J Chemother* 2012;24(2):81-6.

12. Totten PA, Taylor-Robinson D, Jensen JS. Genital mycoplasmas,. *In*: Holmes KK, Sparling PF, Stamm WE, Piot P, Wasserheit JN, Corey L, Cohen MS, and Watts DH. (eds.), Sexually Transmitted Diseases. 4 ed. 2008:709-736. McGraw Hill, New York.

13. Svenstrup HF, Fedder J, Abraham-Peskir J, *et al*. *Mycoplasma genitalium* attaches to human spermatozoa. *Hum Reprod* 2003; 18(10):2103-9.

14. Gdoura R, Kchaou W, Chaari C, et al. *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis* and *Mycoplasma genitalium* infections and semen quality of infertile men. *BMC Infectious Diseases* 2007; 7:129.

For peer review

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

BMJ Open

Unusually low prevalence of Mycoplasma genitalium in urine samples from infertile men and healthy controls – a prevalence study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2014-005372.R1
Article Type:	Research
Date Submitted by the Author:	14-Jul-2014
Complete List of Authors:	Plecko, Vanda; Clinical Hospital Center Zagreb, Department for Clinical and Molecular Microbiology; University of Zagreb, School of Medicine Zele-Starcevic, Lidija; Clinical Hospital Center Zagreb, Department for Clinical and Molecular Microbiology Tripkovic, Vesna; Clinical Hospital Center Zagreb, for Clinical and Molecular Microbiology Skerlev, Mihael; Clinical Hospital Center Zagreb, Department of Dermatology and Venereology ; University of Zagreb, School of Medicine Ljubojevic-Hadzavdic, Suzana; Clinical Hospital Center Zagreb, Department of Dermatology and Venereology ; University of Zagreb, School of Medicine Plesko, Sanja; Clinical Hospital Center Zagreb, Department for Clinical and Molecular Microbiology Marekovic, Ivana; Clinical Hospital Center Zagreb, Department for Clinical and Molecular Microbiology; University of Zagreb, School of Medicine Jensen, Jørgen; Statens Serum Institut, Mycoplasma Laboratory
Primary Subject Heading:	Sexual health
Secondary Subject Heading:	Epidemiology, Infectious diseases, Reproductive medicine
Keywords:	MICROBIOLOGY, EPIDEMIOLOGY, Molecular diagnostics < INFECTIOUS DISEASES

SCHOLARONE™
Manuscripts

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Unusually low prevalence of *Mycoplasma genitalium* in urine samples from infertile men and healthy controls – a prevalence study

Vanda PLECKO[1], Lidija ZELE-STARCEVIC[2], Vesna TRIPKOVIC[2], Mihael SKERLEV[3], Suzana LJUBOJEVIC[3], Sanja PLESKO[2], Ivana MAREKOVIC[1], Jorgen Skov JENSEN[4]

[1] Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb and School of Medicine University of Zagreb, Zagreb Croatia

[2] Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb Croatia

[3] Department of Dermatology and Venerology, Clinical Hospital Centre Zagreb and School of Medicine University of Zagreb, Zagreb Croatia

[4] Mycoplasma Laboratory, Staten's Serum Institute, Copenhagen, Denmark

Corresponding author: Vesna TRIPKOVIC M.D., Ph.D, Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb Croatia. Tel: + 385 1 2367 318; fax: + 385 1 2367 393; e-mail: vbtripkovic@gmail.com

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

ABSTRACT

Objective: The goal of this study was to detect *M. genitalium* in urine samples of infertile men and men without any signs of infection. We wanted to investigate whether *M. genitalium* and other genital mycoplasmas (*M. hominis* and *Ureaplasma* sp.) are found more often in urine samples of infertile men than in asymptomatic controls as well as to determine resistance to macrolides.

Methods: The study included first void urine samples (FVU) taken from 145 infertile and 49 men without symptoms of urethritis. *M. genitalium. C. trachomatis* and *N. gonorrhoeae* were detected by commercial PCR. *T. vaginalis* was detected by microscopy and culture. *M. hominis* and *Ureaplasma* sp. were detected by culture. *M. genitalium* were detected by *in house* PCR, conventional and real-time.

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

Conclusion: Comparing to literature data, unusually low prevalence of *M. genitalium* in infertile men was found. The reasons for such unexpected result could not be documented at this moment; probably local demographic and social characteristics of population could influence the result. So, further studies designed to investigate *M. genitalium* in infertile and other group of patient populations are needed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Strenghts and limitations of this study:

- We are aware of low number of urine samples in both groupes of participants.
- These are only preliminary results, which were unexpected regarding the literature data. These results were confirmed in two independent laboratories, to exclude laboratory error.
- We are planning to continue this study to collect urine samples from larger number of infertile men and compare them with men with urethritis and asymptomatic men as well.
- If the results will be similar (unexpected low number of *M. gentialium*), we will try to determine the reason for such unusually low number of M.g. in our population.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

INTRODUCTION

Reliable detection of *Mycoplasma genitalium* became possible after the development of PCR assays (1,2). The prevalence of *M. genitalium* in patients with non-gonococcal urethritis (NGU) ranges from 13%-42%; and in asymptomatic men from 0% to 15%, respectively. (2,3,4). The impact of *M. genitalium* on male fertility remains unclear (2,5).

Our aim was to detect *M. genitalium, M. hominis* and *Ureaplasma spp.* in FVU samples of infertile and in men without any symptoms and/or sign of infection. Furthermore, we intended to determine the prevalence of macrolide resistance of *M. genitalium*. We restricted our study to infertile men in whom common STIs (*Chlamydia trachomatis, Trichomonas vaginalis, and Neisseria gonorrhoeae)* were excluded.

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

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

METHODOLOGY

The study was approved by the Ethics Committee, 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 filled in a questionnaire regarding reasons of attendance, age, symptoms of urethritis, number of lifetime sexual partners, history of STIs, and recent/current antibiotic treatment.

In total, about 20ml of FVU was taken from the patients; 4-5ml 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 was used. Five ml of 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°C.

C. trachomatis and *N. gonorrhoeae* were detected by PCR (Cobas TaqMan CT/NG Test, v2.0 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). Among the infertile men, 13 were excluded due to infection with a recognised STI (CT, TV, NG), and 3 among the controls, respectively. After the exclusion, a total of 194 FVU samples remained. They were collected in polypropylene containers (Sarstedt, Nümbrecht, Germany) from men who were referred to the Department of Clinical Microbiology and Department of Dermatology, CHC Zagreb. 145 samples were from men as a part of work-up for infertility investigation. None of them had any symptom of

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

genitourinary infections, but all of them had reports of different degree of abnormal semen quality analysis (oligozoospermia, asthenozoospermia, oligoasthenozoospermia). In their female partners tubal factors of infertility were excluded using hysterosonosalpingography (HSSG).

49 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 molecular testing, respectively. Urine samples were centrifuged at 3000x *g* for 5 min and sediments were inoculated in urea-arginin broth (bioMerieux, Lyon, France) and onto A7 agar (Becton Dickinson, Cockeysville, MD, USA). The vials were incubated 48 hours at 37°C. The agar plates were incubated at 37°C in 5% CO_2 for 5 days and examined microscopically for the appearance of typical mycoplasma colonies (6).

The rest of urine (4-5 ml) was concentrated at 20000 x *g* for 15 minutes at +4°C. The pellet was resuspended in 200 µL 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 minute; then placed in a thermoblock for 10 minutes at 95°C. The mixture was centrifuged shortly and the supernatant was aspirated in new tubes and stored at -20°C until PCR was performed (7).

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) (7), 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) (1).

All samples were performed with both assays with internal controls for PCR inhibition and both PCRs were performed as previously described (1,7,8,9).

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

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

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 (7,9).

Macrolide resistance mediating mutations in region V of the 23S rRNA-gene were detected by DNA sequencing of amplicons obtained directly from the clinical specimens, and performed at Statens Serum Institute Copenhagen, Denmark (10).

STATISTICA (data analysis software system), version 10 (StatSoft, Inc. (2011), USA) was used for data analysis. Median was used to describe age of groups and number of observations and percentage to describe categorical variables. To compare age between groups Mann-Whitney U test was used, and to compare categorical variables chi-square test was used. P of <0.05 was considered as statistically significant for all tests performed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

RESULTS

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

Table 1. Demographic and epidemiological data for all groups

	Infertile men N=145	Asymptomatic men N=49
Median age (years)	38	41
History of STIs[a]	81 (55.8%)	12 (24.4%)
No of lifetime partners[b]		
<5	15 (10.3%)	13 (26.5%)
5-10	123 (84.8%)	25 (51.0%)
>10	7 (4.8%)	11 (22.4%)

[a] p=0.0001, Chi-square test

[b] p<0.0001, Chi-square test

Among the infertile men, 13 were excluded due to infection with a recognised STI (CT, TV, NG), and 3 among the controls, respectively.

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

For peer review only

Ureaplasma spp. and *M. hominis* were isolated from the same proportion of infertile men and asymptomatic controls (chi-square=0.435, p=0.509; chi-square=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 0.2-4.9%). These men were 29 and 37 years-of-age, respectively and reported 3 and 7 lifetime sexual partners compared to the majority of the group having 5-10 partners.

In our laboratory we used conventional in-house PCR (qualitative) and results were confirmed in 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 were 778 c/ml and 6765 c/ml, respectively. In the men with *M.genitalium* load of 778 c/ml was diagnosed oligozoospermia, and in the other (*M.genitalium* load of 6765 c/ml) was diagnosed asthenozoospermia.

Ureaplasma spp. was positive in 30% (43/145) of the infertile men compared to 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 twelve samples among infertile men coinfection of *Ureaplasma* spp. and *M.hominis* were found. In the group of the asymptomatic men coinfection in three samples were found. In two samples with positive *M.genitalium*, taken from infertile men, no other pathogens were found.

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

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

DISCUSSION

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

First void urine samples were used because several studies have reported that molecular methods performed on urine samples are able to detect as many or even more infected patients than traditional urethral swabs, or cervical swabs or semen (5,7). 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, being present in approximately one third in both groups. This strongly argues against a significant role of ureaplasmas in male infertility.

We did not perform 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 literature data are not conclusive about the prevalence of *U.urealyticum* and *U.parvum*. In the study of Abusaraha et al. (2013), was found that *U.parvum* was the most prevalent isolate detected among infertile men (90%). (11)

M. hominis was detected in 20% of asymptomatic men and 21% of infertile men, respectively, a higher prevalence than in some other studies (12). *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 sexual partners of the men, as *M. hominis* is known to be strongly associated with this condition in women (13).

The prevalence of *M. genitalium* varies significantly in different populations (2) but was found in at a very low prevalence in the present study. Other studies have also found *M. genitalium* uncommonly in FVU of infertile men (5). The two positive samples were from the

For peer review only

group of 145 infertile men and *M. genitalium* was not detected in any of the controls. We were concerned that technical issues were the reason for 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.

The 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 (5,14). Detection of bacteria in urogenital samples does not necessarily suggest infection because detection of bacteria may signify colonization, contamination or infection.

Only a few studies have examined the association between *M. genitalium* and male infertility (5,15) and these studies suffer from any control populations of fertile men.

We tried to design the study in which all other potential infective causes of infertility were excluded.

In recent studies the prevalence of *M. genitalium* in infertile men were almost similar: 4,8% in the study from Gdoura et al. (2008) and 3,2% was in the study of Abusaraha et al. (2013) (5, 11).

The similar findings are with *C.trachomatis*, which is the most often bacterial cause of NGU. *C.trachomatis* is in women well established cause of tubal factor infertiliy. In men it causes NGU. Also, it is clearly demonstrated that *C.trachomatis* attached to spermatozoa (on the surface and in the nucelous, as well). However, its role in male infertility, as well as the role of *M. genitalium*, is not clear yet. There are significant differences in the prevalence of *C.trachomatis* infections in men with infertility and it varies from 0% to 42,3%, depending of the methodology, type of sample and differences of infection rates in different populations. In the recent published Canadian study (Samplaski et al. 2014), the prevalence of *C.trachomatis*

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

infection studied on 5588 infertile men, was 0,3%. The author concluded that this low prevalence clearly demonstrates that small proportion of male infertility is caused by *C.trachomatis* (16,17).

We attempted to study an asymptomatic group without urethritis as controls, and found all men negative for *M. genitalium*. Unfortunately, the infertile men had significantly more partners and also reported previous STIs more commonly than did the controls suggesting that the control group had less risk-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.

Unusually low percent of *M. genitalium* was found in this study. The reasons for unexpected result could not be explained yet. Further studies designed to investigate *M. genitalium* in infertile and other group of patients from Croatia are needed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Acknowledgements

We wish to thank Birthe Dohn for excellent technical assistance with *M. genitalium* testing at Statens Serum Institut, Copenhagen, Denmark.

Contributorship statement

V.P. has designed the study, was responsible for questionnaire data and data analysis, and wrote the part of the manuscript; L.Z.S. was responsible for testing samples for *M.g,* as well as in drafting of the article; V.T. participated in planning the study and wrote part of the text; M.S. participated in collecting samples and edited the manuscript; S.Lj. participated in collecting samples and questionnaire data; JSJ participated in testing samples for *M.g.* and approved the final version and edited the manuscript; S.P. participated in testing samples and corrected the draft version; I.M. revised the manuscript.

Competing of interest

None declared.

Funding

The study is part of the Croatian Ministry of Science grant (108-1080114-0014): “Molecular detection of microorganisms: their influence on antimicrobial consumption”.

Data sharing statement

No additional data available

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

REFERENCES

1. Jensen JS, Uldum SA, Sondergard-Andersen J, et al. Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J Clin Microbiol* 1991; 29:46-50.
2. Taylor-Robinson D, Jensen JS. *Mycoplasma genitalium*: from Chrysalis to multicolored butterfly. *Clin Microbiol Rev* 2011; 24(3):498-514. doi: 10.1128/CMR.00006-11. Review
3. Ross JDC, Jensen JS. *Mycoplasma genitalium* as a sexually transmitted infection: implications for screening, testing, and treatment. *Sex Transm Infect* 2006; 82; 269-71.
4. Ishihara S, Yasuda M, Ito S, *et al*: *Mycoplasma genitalium* urethritis in men. *Int J Antimicrobial Agents* 2004; 24S:*S23-S27*.
5. Gdoura R, Kchaou W, Ammar-Keskes L, *et al*. Assessment of *Chlamydia trachomatis*, *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis*, and *Mycoplasma genitalium* in semen and first void urine specimens of asymptomatic male partners of infertile couples. *J Androl* 2008; 29(2):198-206. Epub 2007 Dec 12
6. Shepard MC, Lunceford CD. Differential agar medium (A7) for identification of *Ureaplasma urealyticum* (human T mycoplasmas) in primary cultures of clinical material. *J Clin Microbiol* 1976;3:613-625.
7. Jensen JS, Björnelius E, Dohn B, *et al*. Comparison of first void urogenital swab specimens for detection of *Mycoplasma genitalium* and *Chlamydia trachomatis* by polymerase chain reaction in patients attending a sexually transmitted disease clinic. *Sex Transm Dis* 2004; 31(4):499-507.

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

8. Eastick K, Leeming JP, Caul EO, *et al*. A novel polymerase chain reaction assay to detect *Mycoplasma genitalium*. *Mol Pathol* 2003; 56(1):25-8.

9. Jensen JS, Björnelius E, Dohn B, *et al*. Use of TaqMan 5' nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *J Clin Microbiol* 2004;42:683-692.

10. Jensen JS. Protocol for the Detection of *Mycoplasma genitalium* by PCR from Clinical Specimens and Subsequent Detection of Macrolide Resistance-Mediating Mutations in Region V of the 23S rRNA Gene. *Methods in Molecular Biology* 2012;903: 129-139.

11. Abusaraha EA, Awwadb ZM, Charvalosc E, et al. Molecular Detection of potential sexually transmitted pathogens in semen and urine specimens of infertile and fertile males. *Diagnostic Microbiology and Infectious Disease* 2013;77: 283–286.

12. Salmeri M, Valenti D, La Vignera S, et al. Prevalence of *Ureaplasma urealyticum* and *Mycoplasma hominis* infection in unselected infertile men. *J Chemother* 2012;24(2):81-6.

13. Totten PA, Taylor-Robinson D, Jensen JS. Genital mycoplasmas,. *In*: Holmes KK, Sparling PF, Stamm WE, Piot P, Wasserheit JN, Corey L, Cohen MS, and Watts DH. (eds.), Sexually Transmitted Diseases. 4 ed. 2008:709-736. McGraw Hill, New York.

14. Svenstrup HF, Fedder J, Abraham-Peskir J, *et al*. *Mycoplasma genitalium* attaches to human spermatozoa. *Hum Reprod* 2003; 18(10):2103-9.

15. Gdoura R, Kchaou W, Chaari C, et al. *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis* and *Mycoplasma genitalium* infections and semen quality of infertile men. *BMC Infectious Diseases* 2007; 7:129.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

16. Domes T, Lo KC, Grober ED, et al. The utility and cost of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* screening of a male infertility population. *Fertil Steril* 2012;97(2): 299-305.

17. Samplaski MK, Domes T, Jarvi KA. Chlamydial Infection and its Role in Male Infertility. *Advances in Andrology* 2014:vol 2014, Article ID 307950, 11 pages http://dx.doi.org/10.1155/2014/307950.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Unusually low prevalence of *Mycoplasma genitalium* in urine samples from infertile men and healthy controls – **a prevalence study**

Vanda PLECKO[1], Lidija ZELE-STARCEVIC[2], Vesna TRIPKOVIC[2], Mihael SKERLEV[3], Suzana LJUBOJEVIC[3], Sanja PLESKO[2], **Ivana MAREKOVIC[1]**, Jorgen Skov JENSEN[4]

[1] Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb and School of Medicine University of Zagreb, Zagreb Croatia

[2] Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb Croatia

[3] Department of Dermatology and Venerology, Clinical Hospital Centre Zagreb and School of Medicine University of Zagreb, Zagreb Croatia

[4] Mycoplasma Laboratory, Staten's Serum Institute, Copenhagen, Denmark

Corresponding author: Vesna TRIPKOVIC M.D., Ph.D, Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb Croatia. Tel: + 385 1 2367 318; fax: + 385 1 2367 393; e-mail: vbtripkovic@gmail.com

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

ABSTRACT

Objective: The goal of this study was to detect *M. genitalium* in urine samples of infertile men and men without any signs of infection. We wanted to investigate whether *M. genitalium* and other genital mycoplasmas (*M. hominis* and *Ureaplasma* sp.) are found more often in urine samples of infertile men **than** in asymptomatic controls as well as to determine resistance to macrolides.

Methods: The study included first void urine samples (FVU) taken from 145 infertile and 49 men without symptoms of urethritis. *M. genitalium. C. trachomatis* and *N. gonorrhoeae* were detected by commercial PCR. *T. vaginalis* was detected by microscopy and culture. *M. hominis* and *Ureaplasma* sp. were detected by culture. *M. genitalium* were detected by *in house* PCR, conventional and real-time.

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

Conclusion: Comparing to literature data, unusually low prevalence of *M. genitalium* in infertile men was found. The reasons for such unexpected result could not be documented at this moment; probably local demographic and social characteristics of population could influence the result. So, further studies designed to investigate *M. genitalium* in infertile and other group of patient populations are needed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Strenghts and limitations of this study:

- We are aware of low number of urine samples in both groupes of participants.
- These are only preliminary results, which were unexpected regarding the literature data. These results were confirmed in two independent laboratories, to exclude laboratory error.
- We are planning to continue this study to collect urine samples from larger number of infertile men and compare them with men with urethritis and asymptomatic men as well.
- If the results will be similar (unexpected low number of *M. gentialium*), we will try to determine the reason for such unusually low number of M.g. in our population.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

INTRODUCTION

Reliable detection of *Mycoplasma genitalium* became possible after the development of PCR assays (1,2). The prevalence of *M. genitalium* in patients with non-gonococcal urethritis (NGU) ranges from 13%-42%; and in asymptomatic men from 0% to 15%, respectively. (2,3,4). The impact of *M. genitalium* on male fertility remains unclear (2,5).

Our aim was to detect *M. genitalium, M. hominis* and *Ureaplasma spp.* in FVU samples of infertile and in men without any symptoms and/or sign of infection. Furthermore, we intended to determine the prevalence of macrolide resistance of *M. genitalium*. We restricted our study to infertile men in whom common STIs (*Chlamydia trachomatis, Trichomonas vaginalis, and Neisseria gonorrhoeae)* were excluded**.**

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

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

METHODOLOGY

The study was approved by the Ethics Committee, 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 filled in a questionnaire regarding reasons of attendance, age, symptoms of urethritis, number of lifetime sexual partners, history of STIs, and recent/current antibiotic treatment.

In total, about 20ml of FVU was taken from the patients; 4-5ml 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 was used. Five ml of 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°C.

C. trachomatis and *N. gonorrhoeae* were detected by PCR (Cobas TaqMan CT/NG Test, v2.0 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). Among the infertile men, 13 were excluded due to infection with a recognised STI (CT, TV, NG), and 3 among the controls, respectively. After the exclusion, a total of 194 FVU samples remained. They were collected in polypropylene containers (Sarstedt, Nümbrecht, Germany) from men who were referred to the Department of Clinical Microbiology and Department of Dermatology, CHC Zagreb. 145 samples were from men as a part of work-up for infertility investigation. None of them had any symptom of

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

genitourinary infections, but all of them had reports of different degree of abnormal semen quality analysis (oligozoospermia, asthenozoospermia, oligoasthenozoospermia). In their female partners tubal factors of infertility were excluded **using hysterosonosalpingography (HSSG).**

49 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 molecular testing, respectively. Urine samples were centrifuged at 3000x *g* for 5 min and sediments were inoculated in urea-arginin broth (bioMerieux, Lyon, France) and onto A7 agar (Becton Dickinson, Cockeysville, MD, USA). The vials were incubated 48 hours at 37°C. The agar plates were incubated at 37°C in 5% CO_2 for 5 days and examined microscopically for the appearance of typical mycoplasma colonies (6).

The rest of urine (**4-5 ml)** was concentrated at 20000 x *g* for 15 minutes at +4°C. The pellet was resuspended in 200 µL 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 minute; then placed in a thermoblock for 10 minutes at 95°C. The mixture was centrifuged shortly and the supernatant was aspirated in new tubes and stored at -20°C until PCR was performed (7).

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) (7), 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) (1).

All samples were performed with both assays with internal controls for PCR inhibition and both PCRs were performed as previously **described** (1,7,8,9).

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

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

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 (7,9).

Macrolide resistance mediating mutations in region V of the 23S rRNA-gene were detected by DNA sequencing of amplicons obtained directly from the clinical specimens, and performed at Statens Serum Institute Copenhagen, Denmark (10).

STATISTICA (data analysis software system), version 10 (StatSoft, Inc. (2011), USA) was used for data analysis. Median was used to describe age of groups and number of observations and percentage to describe categorical variables. To compare age between groups Mann-Whitney U test was used, and to compare categorical variables chi-square test was used. P of <0.05 was considered as statistically significant for all tests performed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

RESULTS

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

Table 1. Demographic and epidemiological data for all groups

	Infertile men N=145	Asymptomatic men N=49
Median age (years)	38	41
History of STIs[a]	81 (55.8%)	12 (24.4%)
No of lifetime partners[b]		
<5	15 (10.3%)	13 (26.5%)
5-10	123 (84.8%)	25 (51.0%)
>10	7 (4.8%)	11 (22.4%)

[a] p=0.0001, Chi-square test

[b] p<0.0001, Chi-square test

Among the infertile men, 13 were excluded due to infection with a recognised STI (CT, TV, NG), and 3 among the controls, respectively.

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

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

Ureaplasma spp. and *M. hominis* were isolated from the same proportion of infertile men and asymptomatic controls (chi-square=0.435, p=0.509; chi-square=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 0.2-4.9%). These men were 29 and 37 years-of-age, respectively and reported 3 and 7 lifetime sexual partners compared to the majority of the group having 5-10 partners.

In our laboratory we used conventional in-house PCR (qualitative) and results were confirmed in 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 were 778 c/ml and 6765 c/ml, respectively. In the men with *M.genitalium* load of 778 c/ml was diagnosed oligozoospermia, and in the other (*M.genitalium* load of 6765 c/ml) was diagnosed asthenozoospermia.**

Ureaplasma spp. was positive in 30% **(43/145)** of the infertile men compared to 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 twelve samples among infertile men coinfection of *Ureaplasma* spp. and *M.hominis* were found. In the group of the asymptomatic men coinfection in three samples were found. In two samples with positive *M.genitalium*, taken from infertile men, no other pathogens were found.

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

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

DISCUSSION

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

First void urine samples were used because several studies have reported that molecular methods performed on urine samples are able to detect as many or even more infected patients than traditional urethral swabs, or cervical swabs or semen (5,7). 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, being present in approximately one third in both groups. This strongly argues against a significant role of ureaplasmas in male infertility.

We did not perform 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 literature data are not conclusive about the prevalence of *U.urealyticum* and *U.parvum*. In the study of Abusaraha et al. (2013), was found that *U.parvum* was the most prevalent isolate detected among infertile men (90%). (11)

M. hominis was detected in 20% of asymptomatic men and 21% of infertile men, respectively, a higher prevalence than in some other studies **(12)**. *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 sexual partners of the men, as *M. hominis* is known to be strongly associated with this condition in women **(13)**.

The prevalence of *M. genitalium* varies significantly in different populations (2) but was found in at a very low prevalence in the present study. Other studies have also found *M.*

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

genitalium uncommonly in FVU of infertile men (5). The two positive samples were from the group of 145 infertile men and *M. genitalium* was not detected in any of the controls. We were concerned that technical issues were the reason for 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.

The 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 (5,**14**). Detection of bacteria in urogenital samples does not necessarily suggest infection because detection of bacteria may signify colonization, contamination or infection.

Only a few studies have examined the association between *M. genitalium* and male infertility (5,**15)** and these studies suffer from any control populations of fertile men.

We tried to design the study in which all other potential infective causes of infertility were excluded.

In recent studies the prevalence of *M. genitalium* in infertile men were almost similar: 4,8% in the study from Gdoura et al. (2008) and 3,2% was in the study of Abusaraha et al. (2013) (5, **11).**

The similar findings are with *C.trachomatis*, which is the most often bacterial cause of NGU. *C.trachomatis* is in women well established cause of tubal factor infertiliy. In men it causes NGU. Also, it is clearly demonstrated that *C.trachomatis* attached to spermatozoa (on the surface and in the nucelous, as well). However, its role in male infertility, as well as the role of *M. genitalium*, is not clear yet. There are significant differences in the prevalence of *C.trachomatis* infections in men with infertility and it varies from 0% to 42,3%, depending of the methodology, type of sample and differences

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

of infection rates in different populations. In the recent published Canadian study (Samplaski et al. 2014), the prevalence of *C.trachomatis* infection studied on 5588 infertile men, was 0,3%. The author concluded that this low prevalence clearly demonstrates that small proportion of male infertility is caused by *C.trachomatis* (16,17). We attempted to study an asymptomatic group without urethritis as controls, and found all men negative for *M. genitalium*. Unfortunately, the infertile men had significantly more partners and also reported previous STIs more commonly than did the controls suggesting that the control group had less risk-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**.**

Unusually low percent of *M. genitalium* was found in this study. The reasons for unexpected result could not be explained yet. Further studies designed to investigate *M. genitalium* in infertile and other group of patients from Croatia are needed.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

ACKNOWLEDGEMENTS

We wish to thank Birthe Dohn for excellent technical assistance with *M. genitalium* testing at Statens Serum Institut, Copenhagen, Denmark.

Contributorship statement

V.P. has designed the study, was responsible for questionnaire data and data analysis, and wrote the part of the manuscript; L.Z.S. was responsible for testing samples for *M.g,* as well as in drafting of the article; V.T. participated in planning the study and wrote part of the text; M.S. participated in collecting samples and edited the manuscript; S.Lj. participated in collecting samples and questionnaire data; JSJ participated in testing samples for *M.g.* and approved the final version and edited the manuscript; S.P. participated in testing samples and corrected the draft version; I.M. revised the manuscript.

Competing of interest

None declared.

Funding

The study is part of the Croatian Ministry of Science grant (108-1080114-0014): “Molecular detection of microorganisms: their influence on antimicrobial consumption”.

Data sharing statement

No additional data available

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

REFERENCES

1. Jensen JS, Uldum SA, Sondergard-Andersen J, et al. Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J Clin Microbiol* 1991; 29:46-50.
2. Taylor-Robinson D, Jensen JS. *Mycoplasma genitalium*: from Chrysalis to multicolored butterfly. *Clin Microbiol Rev* 2011; 24(3):498-514. doi: 10.1128/CMR.00006-11. Review
3. Ross JDC, Jensen JS. *Mycoplasma genitalium* as a sexually transmitted infection: implications for screening, testing, and treatment. *Sex Transm Infect* 2006; 82; 269-71.
4. Ishihara S, Yasuda M, Ito S, *et al*: *Mycoplasma genitalium* urethritis in men. *Int J Antimicrobial Agents* 2004; 24S:*S23-S27.*
5. Gdoura R, Kchaou W, Ammar-Keskes L, *et al*. Assessment of *Chlamydia trachomatis*, *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis*, and *Mycoplasma genitalium* in semen and first void urine specimens of asymptomatic male partners of infertile couples. *J Androl* 2008; 29(2):198-206. Epub 2007 Dec 12
6. Shepard MC, Lunceford CD. Differential agar medium (A7) for identification of *Ureaplasma urealyticum* (human T mycoplasmas) in primary cultures of clinical material. *J Clin Microbiol* 1976;3:613-625.
7. Jensen JS, Björnelius E, Dohn B, *et al*. Comparison of first void urogenital swab specimens for detection of *Mycoplasma genitalium* and *Chlamydia trachomatis* by polymerase chain reaction in patients attending a sexually transmitted disease clinic. *Sex Transm Dis* 2004; 31(4):499-507.

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

8. Eastick K, Leeming JP, Caul EO, *et al*. A novel polymerase chain reaction assay to detect *Mycoplasma genitalium*. *Mol Pathol* 2003; 56(1):25-8.

9. Jensen JS, Björnelius E, Dohn B, *et al*. Use of TaqMan 5' nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *J Clin Microbiol* 2004;42:683-692.

10. Jensen JS. Protocol for the Detection of *Mycoplasma genitalium* by PCR from Clinical Specimens and Subsequent Detection of Macrolide Resistance-Mediating Mutations in Region V of the 23S rRNA Gene. *Methods in Molecular Biology* 2012;903: 129-139.

11. Abusaraha EA, Awwadb ZM, Charvalosc E, Shehabi AA. Molecular Detection of potential sexually transmitted pathogens in semen and urine specimens of infertile and fertile males. *Diagnostic Microbiology and Infectious Disease* 2013;77: 283–286.

12. Salmeri M, Valenti D, La Vignera S, et al. Prevalence of *Ureaplasma urealyticum* and *Mycoplasma hominis* infection in unselected infertile men. *J Chemother* 2012;24(2):81-6.

13. Totten PA, Taylor-Robinson D, Jensen JS. Genital mycoplasmas,. *In*: Holmes KK, Sparling PF, Stamm WE, Piot P, Wasserheit JN, Corey L, Cohen MS, and Watts DH. (eds.), Sexually Transmitted Diseases. 4 ed. 2008:709-736. McGraw Hill, New York.

14. Svenstrup HF, Fedder J, Abraham-Peskir J, *et al*. *Mycoplasma genitalium* attaches to human spermatozoa. *Hum Reprod* 2003; 18(10):2103-9.

15. Gdoura R, Kchaou W, Chaari C, et al. *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis* and *Mycoplasma genitalium* infections and semen quality of infertile men. *BMC Infectious Diseases* 2007; 7:129.

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.

16. **Domes T, Lo KC, Grober ED, Mullen JB, Mazzulli T, Jarvi K. The utility and cost of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* screening of a male infertility population. *Fertil Steril* 2012;97(2): 299-305.**

17. **Samplaski MK, Domes T, Jarvi KA. Chlamydial Infection and its Role in Male Infertility. *Advances in Andrology* 2014:vol 2014, Article ID 307950, 11 pages http://dx.doi.org/10.1155/2014/307950.**

For peer review only

BMJ Open: first published as 10.1136/bmjopen-2014-005372 on 25 August 2014. Downloaded from http://bmjopen.bmj.com/ on June 30, 2024 by guest. Protected by copyright.