Prevalence of *Mycoplasma genitalium* and other sexually-transmitted pathogens among high-risk individuals in Greece

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Abstract

Background **The aim of the present study was to determine the prevalence of *Mycoplasma genitalium* (MG) infection among individuals at high risk for sexually-transmitted diseases (STDs) at a major urban STD clinic in Athens, in view of the lack of data pertaining to this infection in Greece.**

Methods **Urethral and cervical samples from 176 individuals consecutively attending the clinic and agreeing to participate were prospectively collected and tested for MG infection using conventional PCR and TaqMan Real-Time PCR. All individuals were also examined for alternative STD pathogens.**

Results **A total of 161 individuals (91.5%) reported symptoms, while 15 individuals (8.5%) were asymptomatic. MG was detected in 5.7% (10/176) of the total population and in 5.6% (9/161) of those with symptoms, corresponding to 5.7% (5/87) of symptomatic men and 5.4% (4/74) of symptomatic women. Among symptomatic males, 3.4% (3/87) displayed MG mono-infection. The median age of MG infected individuals was 25 years (IQR 21.5-29.5 years). Individuals infected with MG were more likely to be coinfected with *Ureaplasma* spp. [OR=5.12, 95%CI, 1.27-20.57] (p=0.017). MG infection was also more common among individuals who had received antibiotics in the previous 15 days [OR=6.04, 95%CI, 1.37-26.64] (p=0.035).**

Conclusion **MG was found to represent an important microbial pathogen among patients presenting with symptoms of urethritis or cervicitis in Greece. Consideration of MG as cause of STD seems crucial in diagnostic algorithms and treatment strategies.**

Keywords ***Mycoplasma genitalium*, prevalence, coinfection, molecular diagnosis.**

Introduction

Mycoplasma genitalium (MG), which was first isolated in 1980,[1] represents the smallest free-living self-replicating organism, with the smallest genome size among all known mycoplasmas.[2] As all mycoplasmas, MG lacks a rigid cell wall and is non-susceptible to beta-lactams and other antibiotics that target the cell wall.[3] Due to its serologic cross-reactivity and morphologic similarities with M. *pneumoniae*,[4] along with its extremely fastidious growth requirements,[1] the study of MG is challenging, but has made substantial progress after the introduction of PCR assays.[5,6]

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MG is recognized as a sexually-transmitted pathogen.[7,8] It has been identified as a leading cause of acute and persistent non-gonococcal urethritis (NGU) in men and has also been associated with prostatitis, epididymitis, balanoposthitis, human immunodeficiency virus (HIV) transmission and proctitis.[9] In women, MG infection has been associated with urethritis, cervicitis, pelvic inflammatory disease, susceptibility to HIV, adverse pregnancy outcomes and infertility.[10] Through intracellular localization[11] and antigenic variation,[12] MG could result in treatment-resistant chronic infection.[10]

Molecular assays are currently employed, with high sensitivity, as the major diagnostic techniques for MG infection, although, at present they rely only on in-house protocols, as FDA-approved ones are not available.[13] Considering the increasing evidence of adverse sequelae associated with MG infection and the lack of data pertaining to MG as a sexually-transmitted disease (STD) agent among high-risk individuals in Greece, we performed a prospective study among symptomatic and asymptomatic adult men and women, presenting to a major urban STD clinic in Athens, Greece, to assess prevalence and risk factors for MG infection.

Methods

Study design

Samples for this study were collected from consenting participants at the STD outpatient department of "Andreas Syggros" Hospital, Athens, during a 16-month period (September 2010-February 2012). Included in the study were all symptomatic adult males and females and all asymptomatic adult males and females seeking to exclude an STD. Classified as "symptomatic" were men reporting clinical manifestations of urethritis (urethral discharge and/or dysuria and/or urethral pruritus), and women reporting clinical manifestations of cervicitis (abnormal vaginal discharge, vaginal bleeding after sexual contact, dysuria). Urethritis was defined as urethral discharge and/or dysuria, and cervicitis was defined as purulent or mucopurulent cervical discharge and/or easily induced cervical bleeding (friability).[14]

Individuals with genital lesions, females that were examined within 10 days of their menstrual cycle, with a history of hysterectomy, women who had an intrauterine device and pregnant women, were excluded from the study. Also excluded were participants unable to understand a standardized questionnaire with demographic, epidemiological and clinical data.

Ethics approval

The study was approved by the Bioethical Committee of the National and Kapodistrian University of Athens (approval no. 10954, 30/09/2009). Informed consent was obtained from all study participants prior to inclusion in the study. The informed consent and the standardized questionnaire for the study were also approved by the Bioethical committee. All data were evaluated in a fully anonymous way.

Epidemiological and clinical data

Using a standardized questionnaire, the following clinical data were collected: clinical symptoms on presentation (urethritis, vaginitis and cervicitis), presence and duration of vaginal/cervical discharge or urethral discharge, dysuria, bleeding after sexual contact. In addition, the following demographic and epidemiological data were recorded: age, gender, profession, place of residence, nationality, marital status (single, married or divorced), age of first sexual contact, age of first menstruation, contraceptive use, condom use, sexual orientation, number of sexual partners and antibiotics taken in the three months preceding the examination, alcohol consumption, tobacco and drug use, HIV infection, and history of other STDs.

Specimen collection

Urethral swab specimens were collected from all male patients, and vaginal and endo-cervical swab specimens were collected from all female patients. The urethral specimen was obtained by inserting a thin Dacron-tipped sterile swab 1-2 cm within the urethra and rotating it for 2-4 seconds. In women, a sterile speculum was placed in the cervix and the sample was taken

from the posterior fornix of the vagina/vaginal walls, and the endocervix.

A first void urine sample in a sterile plastic 50 mL screw-cap, at least 2 hours after the last urination, was also provided by each participant, and was subsequently divided into two parts.

Diagnostic microbiology

A first swab specimen (urethral from men and vaginal from women) was used for direct microscopy and conventional culture. Urethritis was determined microscopically by the presence of ≥5 white blood cells (WBC) per 1000x microscopic field in urethral specimens, whereas cervicitis was determined in symptomatic cases by the presence of >10 WBC per high power field on microscopic examination of vaginal fluid.[14] *Trichomonas vaginalis* (TV) was actively sought and noted by light microscopy. *Candida* (C) was detected by wet-smear microscopy and/or culture. Gram-stain was performed to determine bacterial flora and possible presence of Gram–negative intracellular diplococci. Bacterial vaginosis (BV) was diagnosed using Amsel's criteria[15] and after microscopic evaluation of the Gram-stained specimens.

A second swab specimen, urethral from men and endocervical from women, was used for: a) culture of *Neisseria gonorrhoeae* (NG) by inoculation of the specimen on a modified Thayer-Martin agar plate and incubation for 48 hours at 37 °C and 5% CO_2, b) detection of *Ureaplasma* spp. (UU) and *Mycoplasma hominis* (MH) using the *Mycoplasma* Duo Kit (Bio-Rad Laboratories M E.P.E., Athens, Greece).

A third swab specimen (urethral from men and endocervical from women) and the first half of the urine sample were used for molecular detection of MG and *Chlamydia trachomatis* (CT). The swab specimen from the endocervix was obtained after first removing cervical secretions and mucus with a sterile swab. All swab specimens were diluted in 2SP medium, vortexed, centrifuged for 20 minutes at 5000 rpm (6800 x g) and the pellet was used for DNA extraction using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and the tissue protocol, according to the manufacturer's instructions. The urine specimens were also centrifuged for 20 minutes at 5000 rpm (6800 x g) and the pellet was used for DNA extraction using the same methodology.

MG was detected using a conventional PCR protocol targeting the V1/V3 hypervariable regions of the 16S rRNA gene,[16] which is one of the three genes in the single MG rRNA operon,[2] as well as an MGB TaqMan Real-Time PCR assay,[17] targeting a single copy region of the MgPa operon, as described previously.[18] Two different PCRs were used, with different gene targets, to ensure that the positive results with both PCRs were true positive and not contamination (stringent criteria). The primers used were MG16-45F and MG16-447R, as previously described.[16] The parameters were those described by Jensen et al.[17] True positive cases were defined as a positive result in at least one of the two specimens collected from each patient (urine or swab) by both PCR assays. PCR for the beta-actin gene (as internal positive control of both PCRs) was performed on random specimens (approximately 30%) and no inhibition was detected. CT was detected using the commercially available Cobas Amplicor PCR platform (Roche Molecular Systems Inc., Branchburg, NJ, USA) according to the manufacturer's instructions.

The second half of the urine specimen was used for urinalysis and microscopic examination.

Statistical evaluation

The description of data was made using techniques such as medians along with 25% and 75% percentiles in the case of continuous variables and percentages in the case of categorical variables. The Chi-squared test was used for assessing independence between two categorical variables. When the expected numbers in the association table were small, Monte Carlo was used to determine the p value of the Chi-square criterion, or in the case of 2×2 tables, Fisher's exact test. Logistic regression was used for estimating the odds ratios of MG infection between groups and for determining the degree of association with continuous variables. The effect of every possible explanatory variable, either continuous or

categorical was tested as a single step procedure without any attempt to fit an overall model due to the small sample size. The significance level for all tests was set to 5%. All calculations were performed with IBM SPSS statistics v.24 (IBM Corp., Armonk, NY, USA).

Results

Characteristics of the study population

A total of 176 individuals (94 (53%) males and 82 (47%) females) were consented and included in the study. These individuals presented to the STD outpatient department with symptoms of urethritis/cervicitis or for sexually-transmitted infection screening due to high-risk social history. Of the 94 males, 11 (11.7%) identified themselves as homosexual and 5 (5.3%) as bisexual. None of the women identified herself as homosexual or bisexual. The median age of the study population was 29 years (IQR 23-38 years). The median ages of males and females were 28.5 years (IQR 23-36.2 years) and 31 years (IQR 23-42 years), respectively.

Among the 176 studied participants, 161 (91.5%) had clinical symptoms, while the remaining 15 (8.5%) were asymptomatic but at risk for STD. The median ages of symptomatic and asymptomatic participants were 29 years (IQR 23-37 years) and 34 years (IQR 27-45 years), respectively. Symptomatic male and female patients corresponded to 49.4% and 42% of the study population, respectively, or to 54% and 46% of symptomatic individuals. Asymptomatic male and female patients corresponded to 4% and 4.5% of the total study population respectively, and to 46.7% and 53.3% of total asymptomatic individuals (Table 1). Among males and females, 83% (78/94) and 26.8% (22/82) displayed microscopic findings of urethritis and cervicitis, respectively.

Table 1. Asymptomatic and symptomatic individuals of the study population

	Asymptomatic no. (%) of participants	Symptomatic no. (%) of participants	Total no. (%) of participants
Men	7 (7.4)	87 (92.6)	94 (100)
Women	8 (9.8)	74 (90.2)	82 (100)
Total	15 (8.5)	161 (91.5)	176 (100)

M. genitalium prevalence and specific characteristics of the infected patients

MG was detected in 5.7% of all persons included in the study, corresponding to 6.4% among males, and to 4.9% among females. It should be noted that no discrepancies were observed between the two different PCR assays. The median age of MG infected persons (MGIP) was 25 years (IQR 21.5-29.5 years). The median ages of males and females were 26.5 years (IQR 24.5-30.7 years) and 21 years (IQR 20-28.7 years), respectively.

The mean age for first sexual contact among MGIP was 17.4 years and the median age 17.5 years (IQR 16-19 years). The corresponding mean/median age for males was 17.5 years/17 years (IQR 16-18.2 years), whereas for females it was 17.3 years/18 years (IQR 17-19 years).

Among symptomatic patients, the pathogen was detected in 5.6% of cases, corresponding to 5.7% of symptomatic men and 5.4% of symptomatic women. Among asymptomatic individuals, MG was detected in one male. Among the MG infected males, 83.3% (5/6) had microscopic findings, symptoms and signs of urethritis. Three of those were MG mono-infected (without coinfection). All of the MG infected females (4/4) were symptomatic, 75% (3/4) displayed symptoms and signs of cervicitis, and 25% (1/4) had symptoms, signs and microscopic findings of cervicitis. MG was recovered from both the swab and urine specimens in 5/6 of the infected males and in 2/4 infected females. A summary of the main characteristics for patients diagnosed with MG infection can be seen in Table 2 (shown in the appendix).

Factors associated with *M. genitalium* infection

The number of MGIP who had taken antibiotics (mainly amoxicillin, with or without clavulanate) within the last 15 days prior to examination was greater than that of those who had not taken antibiotics (21.4% versus 4.3%) (p=0.035; OR=6.04, 95%CI, 1.37-26.24) (Table 3). A frequency of epithelial cells in urine ≥7/hpf was observed among MGIP (p=0.043).

Table 3. Proportions of *M. genitalium* occurrences at different levels of statistically significant predictors, along with p-values of predictor effects.

Predictor	Levels	Sample size	Proportion MG (%)	OR [95%CI]	p-value
Nationality	Greek	139	3.6		0.015
	Balkans	18	16.7	5.36 [1.16-24.69]	
	Ex-Union of Soviet Socialist Republics	7	0.0	0.0	
	Middle-East	6	16.7	5.36 [0.52-54.83]	
	Asia	5	0.0	0.0	
	Africa	1	100.0	>100.0	
Epithelial cells /hpf in urine	1-2	93	5.4	0.15 [0.03-0.75]	0.043
	3-4	41	2.4	0.07 [0.01-0.73]	
	5-7	31	3.2	0.09 [0.01-0.97]	
	≥7	11	27.3		
Antibiotics taken (last 15 days)	No	162	4.3		0.035
	Yes	14	21.4	6.04 [1.37-26.64]	

Odds ratios [95%CI in parenthesis] are also presented comparing odds of *M. genitalium* occurrence between each level and a reference level.

No statistical association between MG infection and microscopically defined urethritis (p=1.0) or microscopically defined cervicitis (p=1.0) was found. Age, gender, location of residence, smoking, alcohol, sexual orientation, HIV infection, number of sexual partners, condom use, WBC in urine and epithelial cells in swab, were not statistically associated with MG infection.

Coinfections associated with *M. genitalium*

Of the 176 participants, 1.7% (3/176) displayed only MG infection without coinfection, all symptomatic men. All other MGIP (7/176; 4.0%) showed combinations of detected STDs (Table 4).

The percentage of MG occurrence with UU presence was 11.9%, versus 2.6% with UU non-presence (p=0.017; OR=5.12, 95%CI, 1.27-20.57). A marginally insignificant coinfection trend with BV (percentage of MG occurrence 25.0% with BV presence versus 4.8% with BV non-presence) was also observed (p=0.068; OR=6.04; 95%CI, 1.37, 26.64).

Discussion

The present study sought to assess the prevalence of MG in a population of adult men and women at STD risk in Greece. Using a group of patients with symptoms of urethritis/cervicitis or at risk for STD, we were able to address risk factors for MG infection and to review these findings in the context of previously published pertinent data.

The overall prevalence of MG infection in our study was 5.7%, similar to 5.9% reported in France,[13] but higher than the 3.4% recently reported MG prevalence from 16 French university hospitals.[19] A 6.4% prevalence among males in our study and a 4.9% prevalence among females are in accordance with 6.6% reported prevalence of MG infection among symptomatic males in Madrid[20] and Tel Aviv[21] and 4.5% among females in a corresponding population in Norway.[22]

When taking into account only symptomatic patients in our study, MG prevalence was 5.7% and 5.4% among males and females, respectively. A recent study from

Table 4. *M. genitalium* infected individuals with corresponding coinfections

Gender	Symptoms	STD	% (n/10)
Male	Yes	MG only	30 (3/10)
Male	No	MG + UU	10 (1/10)
Male	Yes	MG + UU	10 (1/10)
Female	Yes	MG + UU	20 (2/10)
Male	Yes	MG + UU + MH + NG	10 (1/10)
Female	Yes	MG + UU + MH + BV	10 (1/10)
Female	Yes	MG + UU + CT + BV	10 (1/10)

BV – bacterial vaginosis; CT – *C. trachomatis*; MG – *M. genitalium*; MH – *M. hominis*; NG – *N. gonorrhoeae*; UU – *Ureaplasma* spp.

central Greece has reported absence of MG infection among individuals referred and investigated specifically for infertility problems.[23]

However, other studies from Europe, Australia, New Zealand, USA, Asia and Africa have revealed a greater prevalence among STD clinic attendees, whether symptomatic or asymptomatic, in the range of 8.1% to >50%.[24]

In the present study, MG as mono-infection was detected in three symptomatic males, all with microscopic findings of urethritis. This evidence, along with the fact that no simultaneous coinfection with CT or NG was found, supports the idea that MG independently contributes to pathogenicity[25] and should be included in routine testing for STIs in clinical practice.

Among the seven remaining MGIP, UU was the only common denominator in coinfection cases; the observation that 40% of MG isolates were found in dual combination with UU and that members of the Mycoplasmataceae family (MH and UU) were co-isolated with MG may indicate how members of this family tend to be isolated in combinations, suggesting the possibility of common risk factors or a susceptibility link.

Condom use was not significantly protective against acquisition of MG infection in our study (p=0.72). The absolute majority of all MGIP were single (9/10 patients not married) and a significant proportion of MG cases (60%) were detected in smokers. These observations are referring to lifestyle and life habits, which may be related to sexual behavior and practices associated with STD acquisition.

Participants responded to the question of whether or not they had received antibiotics for any reason over a specified time period. The association between antibiotic use and MG infection may reflect either the fact that MG was not hampered due to the lack of efficacy of an antibiotic administered to combat a non-STD medical condition, or due to an inappropriate choice of antibiotic in the quest of treating clinical manifestations of urethritis or cervicitis.

A high number of sexual partners increases the risk of infection with sexually transmitted pathogens. However, in our study, three of the four infected unmarried females and one married male, all reporting only one sexual partner in the previous three months before the examination, were symptomatic. Further, one of the two infected individuals reporting more than seven sexual partners and inconsistent condom use, was asymptomatic. All results mentioned above may indicate that both MG infection and clinical manifestations can be related to chronic infections (longer than three months old), or can be attributed to sequential sexual partners over time, particular sexual practices employed, as well as longstanding inter-sexual/inter-partner networks engaged in by individuals evaluated for STDs.

Study limitations and strengths

Limitations of this study include the inability to recruit larger numbers of asymptomatic individuals; this could have weakened the analyses of association. The results reflect the epidemiology of STD in a particular geographic location, and may not be generalized to all patient populations. Non-microbiological data were gathered as part of a questionnaire, in which participants may have been hesitant to reveal information that they may view as considered socially unacceptable.

For the isolation of UU, the *Mycoplasma* Duo kit, which detects *Ureaplasma* spp. (i.e. *U. parvum*–biovar 1 and *U. urealyticum*-biovar 2) and cannot differentiate between serovars was utilized. We do not know the extent to which our study population harbored *U. urealyticum*-biovar 2 and *U. parvum*-biovar 1.

The study's strengths include the prospective character of the study, the concurrent investigation for seven sexually-transmitted microorganisms and BV occurrence with the possibility of detecting coinfections, the utilization of microscopic investigation which confirms urethritis/cervicitis, and the performance of cultures for NG, which can provide information regarding susceptibility to antibiotics. Finally, to the best of our knowledge, this is the first study in Greece to document the prevalence and characteristics of MG among high-risk men and women.

Conclusions

In conclusion, the overall MG prevalence in our study population is on the lower end of the European/international studies. The present study also revealed a statistically significant association between MG isolation and antibiotic use, mainly amoxicillin-based. Younger age showed greater vulnerability, and the majority of the infected persons were smokers. Multiple sexual partners and use of condoms did not seem to have a differential effect on isolation frequency. No dual infection of MG with CT or NG was found in our study. UU was the only common denominator for coinfection, with 40% of MG isolates in dual coinfection with UU. Taking into account the different prevalence of MG infection between Greek and non-Greek nationals, the higher prevalence rates reported in a number of other populations and the rapid change in the fabric of our population due to current immigration patterns, inclusion of MG in routine STD screening should thus be considered. Since MG infection was variably diagnosed with either a urine specimen or urethral/cervical swab specimen, it may be necessary to routinely obtain both samples in females with suspected infection.

Note: Part of this work has been presented at the 25th ECCMID, April 2015, (P0353).

Authors' contributions statement: PC, JP, EP, GA and AT designed, interpreted the results and wrote the manuscript. PC, JP, EP, GP participated in data acquisition, literature search and laboratory work. PC and ML participated in statistical analysis; PC, JP, and AT were involved in critical revision of the manuscript. All authors read and approved the final version of the manuscript.

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Appendix

Table 2. Characteristics of patients with positive PCR results of *M. genitalium*

Patient no.	Gender	Sexual orientation	Age	Marital status	Smoker	Condom use	Sexual partners no.	Age of first sexual contact (years)	Ethnicity	Positive swab (S) or urine (U) specimen	Symptoms (duration in days)	Antibiotic use (days prior to examination when last dose taken)
1	Male	Heterosexual	28	Not married	Yes	Always	7	15	Gr	S	None	No
2	Male	Heterosexual	29	Not married	Yes	Always	2	17	Gr	S + U	Dysuria (30) Discharge (30)	Amoxicillin (10)
3	Male	Heterosexual	23	Not married	Yes	Inconsis-tent	3	15	Syr	S + U	Dysuria (4) Discharge (3)	No
4	Male	Homosexual	25	Not married	No	Always	2	18	Gr	S + U	Dysuria (10) Discharge (10)	Yes (15)
5	Male	Heterosexual	25	Not married	No	Always	2	17	Alb	S + U	Dysuria (7) Discharge (30)	Amoxicillin+ clavulanate (7)
6	Male	Heterosexual	36	Married	Yes	Never	1	19	Gr	S + U	Dysuria (14)	No
7	Female	Heterosexual	20	Not married	Yes	Always	8	14	Rom	S + U	Dysuria (60) Discharge (>60)	No
8	Female	Heterosexual	33	Not married	Yes	Never	1	13	Gr	S	Discharge (7)	No
9	Female	Heterosexual	20	Not married	No	Never	1	14	Alb	S + U	Dysuria (14) Discharge (7)	No
10	Female	Heterosexual	22	Not married	No	Never	1	18	Nig	U	Dysuria (30) Discharge (30)	Amoxicillin (>30-<90)

Alb – Albanian; Gr – Greek; Nig – Nigerian; Rom – Romanian; Syr – Syrian