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ORIGINAL PAPER

Simultaneous detection and identification of STI pathogens by multiplex Real-Time PCR in genital tract specimens in a selected area of Apulia, a region of Southern Italy

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Abstract
Purpose Genital tract infections are globally a major cause of morbidity in sexually active individuals. The aim of this study was to investigate the prevalence and associations of co-infections of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Mycoplasma hominis* (MH), *Mycoplasma genitalium*, *Ureaplasma urealyticum* (UU) and *Ureaplasma parvum* (UP) in specimens collected from female (SF) and male (SM) patients.
Methods 1575 samples from 1575 individuals from the geographical area around Bari, Apulia region in Southern Italy, were collected and analyzed by a multiplex Real-Time PCR (mRT-PCR) (Anyplex™ II STI-7, Seegene, Inc., Seoul, Korea) assay.
Results 455/1575 (28.89%) samples resulted positive for at least one of the targets named above. Statistically significant differences in prevalence of the pathogens between SF and SM were not detected except for UP (24.92% in SF vs 8.91% in SM). Prevalence of co-infections was 6.84 and 3.96% in SF and SM, respectively. Moreover, MH presence in SF, but not in SM, was associated with UU and UP.
Conclusions Our data suggest different patterns of infections between females and male and the importance of an increased vigilance of sexually transmitted pathogens to reduce the burden on general population and the sequelae or the complications on reproductive organs.

Keywords Genital tract · Sexually transmitted infections · Co-infections · Multiplex Real-Time PCR

Introduction

Worldwide, sexually transmitted infections (STIs) represent a major global health problem with 340 million of cases every year [1]. STIs are caused by different microorganisms including bacteria, viruses, protozoa and mycetes and are associated with acute illness, infertility and long-term sequelae in upper genital tract [2]. Among bacterial pathogens, *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) are the most common causes of STIs [3]. WHO estimates that over 90 million new cases of CT infections and 106 million of gonococcal diseases occur globally each year [4].

Mollicutes (*Mycoplasma spp.* and *Ureaplasma spp.*) are associated with genital tract infections, being potential agents of maternal, fetal and neonatal infections or co-factors in contributing to adverse pregnancy outcomes. The role of *Mycoplasmas* in either female urogenital infections or their complications has been demonstrated [5]. Organisms of this genus are indeed associated with bacterial vaginosis, pelvic inflammatory disease, preterm labor and preterm birth [5]. Among members of genital *Mycoplasmas, Mycoplasma genitalium* (MG) and *Mycoplasma hominis* (MH) are emerging as important causative agents of sexually transmitted infections in both males and females, leading to infertility [6]. In particular, MH plays an important role in developing infertility by creating asymptomatic

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infections that lead to not referring to the physician and the disease progression [7].

Currently, *Ureaplasma* spp. are separated into two species: *Ureaplasma urealyticum* (UU) and *Ureaplasma parvum* (UP). Both the microorganisms have been considered commensals of the uro-genital tract [8]. Meanwhile, several studies have reported that UU is associated with some diseases including non-gonococcal urethritis (NGU), pregnancy complications and prenatal infections [9]. UP is an overlooked pathogen and its pathogenetic role has not yet been demonstrated, although it has been reported that *Ureaplasma* genus may perturb homeostasis in the genital tract providing a survival advantage for CT [8, 9]. Finally, the protozoan *Trichomonas vaginalis* (TV) causes trichomoniasis, the most common non-viral sexually transmitted infection, all over the world. Since trichomoniasis is mostly asymptomatic, it remains poorly diagnosed [10].

Generally, STIs are often asymptomatic or responsible for non-specific symptoms: therefore, if undiagnosed, they will lead to reproductive sequelae or complications in the upper genital tract. On this basis, a sensitive and affordable method for identifying pathogens in clinical samples is needed [11]. In this regard, nucleic acid amplification tests (NAATs) have been shown to be more sensitive than the previously diagnostic tests (i.e., culture, antigen detection and nucleic acid hybridation) [12, 13].

Patients and methods

From June 2014 to February 2016, 1575 specimens from 1575 subjects (303 males and 1272 females, male to female ratio = 0.25) from the geographical area around Bari, Apulia region, Southern Italy, were collected and processed in the Laboratory of Molecular Biology, U.O.C. Microbiology and Virology, Azienda Ospedaliero-Universitaria, Policlinico of Bari, Italy. In particular, specimens included 1167 cervical swabs, 105 vaginal swabs, 177 seminal fluids, and 126 male urethral swabs. Swabs were obtained using a commercial rigid cotton-tipped swab applicator (Nuova Aptaca, Cannelli, Italy). Specimens were transported without added transport medium and examined within 48 h. Otherwise, they were stored at −20 °C until processing. Sample informations (date of sampling, ward, type of specimen, final testing results) together with the data of patients for whom molecular testing was performed (i.e., age and sex) were recorded in an anonymous database by changing sensitive data into alphanumeric codes. No clinical data associated with these specimens were available. As retrospective study, formal consent is not required.

Treatment of clinical specimens

To collect bacterial cells from swabs specimens, 1 mL of phosphate buffered saline (pH 7.4) (Sigma-Aldrich, Milano, Italy) was added to each sample and the tubes were mixed by vortexing. To concentrate the samples, 1 mL of all the mixed specimens was transferred to 2 mL microcentrifuge tubes and the tubes were centrifuged at 15,700*g* for 15 min at 7 °C. The supernatant was discharged and pellet was resuspended in 200 µl of phosphate sodium buffer medium (Carlo Erba, Milano, Italy).

Extraction of bacterial DNA

DNA was extracted using MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics GmbH, Mannheim, Germany) and performed on the MagNa Pure Compact System (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 200 µL of resuspended samples was added to 180 µL of Bacterial Lysis Buffer (Roche Diagnostics GmbH, Mannheim Germany) along with 10 µL of Proteinase K (Roche Diagnostics GmbH, Mannheim Germany). To check the whole process, from the nucleic acid extraction up to multiplex Real-Time PCR (mRT-PCR), 10 µL of internal control (IC), included in the amplification kit, was added to the mixture. From 400 µl of mixture's starting volume, after the extraction phase, the final elution obtained volume was 50 µl.

mRT-PCR

DNA amplification was performed by Anyplex™ II, STI-7 Detection Kit (Seegene, Inc., Seoul, Korea), according to the manufacturer's instructions. Test permits amplification, detection and differentiation of seven microorganisms target DNA including CT, NG, TV, MG, MH, UU and UP [14]. From the DNA elution final volume of 50, 5 µL was tested by the Anyplex™ II STI-7 Kit. mRT-PCR was performed by the CFX96 Real-Time System (Biorad Laboratories, California, CA, USA).

Statistical analysis

Independence of categorical variables was assessed by two-tailed Fisher's exact test as appropriate. Median, interquartile range and differences of age were evaluated

on 1099 female and 243 male patients, respectively, because of the unavailability of some birth dates.

To evaluate the differences in median age between female and male patients, a randomization test on 100,000 random replicates of 243 samples selected from the 1099 female patients without replacement was performed. For each replicate, a one-way Mann–Whitney test and its *p* value were evaluated. After 100,000 random replicates, final p value was calculated as 1 − [(*p* values <0.05)/(number of replicates + 1)].

Differences in prevalence of CT, NG, UU, UP, MH, MG, TV and co-infections between samples collected from female and male patients were evaluated by Odds ratios and double-way Fisher's exact test with p values corrected for multiple comparison by Benjamini and Hochberg's (BH) procedure with false discovery rate (FDR) <5% [15]. However, to more precisely compare different size populations (1272 vs 303 samples), randomization tests on 100,000 random replicates of 303 samples selected from samples collected from female patients dataset without replacement were also performed.

In particular, for each replicate, statistical differences in prevalence for the seven microorganisms and presence of co-infections were evaluated by Fisher's exact test and BH's correction. After 100,000 random replicates, final p values were calculated as 1 – [(BH adjusted *p* values <FDR)/(number of replicates + 1)].

Evaluation of association of different microorganisms in samples collected from female and male patients, respectively, was performed by univariate and multivariate analysis.

In particular, double-way Fisher's exact test was performed to evaluate independence of every couple of microorganisms and p values were corrected for multiple comparisons by BH's procedure. Moreover, a logistic regression model was evaluated for each microorganism and p values were also adjusted by BH's procedure. To verify consistency of results, 100,000 random replicates of 890 and 212 samples were extracted without replacement from datasets of samples collected from female and male patients, respectively. Fisher's test on every couple of microorganisms with BH's correction and logistic regression models with BH's correction of p values were evaluated on every replicate. At least, final p values were calculated as 1 − [(BH adjusted p values <FDR)/(number of replicates + 1)].

Calculations of all statistical tests were performed by the open source statistical software R (version 3.2.4) [16].

A *p* value <0.05 was considered statistically significant.

Results

From June 2014 to February 2016, 1272 specimens (1167 cervical swabs and 105 vaginal swabs) from 1272 female patients (SF) and 303 specimens (177 seminal fluids and 126 urethral swabs) from 303 male patients (SM) were collected and analyzed.

Median age of female and male patients was 34.30 [interquartile range (IQR): 29.03–39.53] and 38.31 (IQR: 32.64–46.83), respectively. Median age of male patients was significantly higher than that of female patients [*p* value after 100.000 random replicates ($p_{100.000}$) <0.001].

391/1272 (30.74%) specimens collected from female patients (SF) were positive for DNA for at least one microorganism. In particular, 304 (23.90%) samples were positive for one microorganism, 64 (5.03%) for two, 18 (1.42%) for three, 4 (0.31%) for four and 1 (0.07%) for five microorganisms. Overall, 23 samples were positive for CT DNA (1.81%), 2 for NG DNA (0.16%), 66 for UU DNA (5.19%), 317 for UP DNA (24.92%), 75 for MH DNA (5.90%), 5 for MG DNA (0.39%) and 19 for TV DNA (1.49%), respectively. Prevalence of co-infections was 6.84% (87 samples)

Table 1 Prevalence of the seven microorganisms detected by Anyplex™ II STI-7 kit in samples collected from female and male patients, respectively

Microorganisms	SF (1272 samples), *N* (%)	SM (303 samples), *N* (%)	Odds ratio (95% CI) SF vs SM	*p* value	BH *p* value	$p_{100.000}$
CT	23 (1.81)	11 (3.63)	0.49 (0.23–1.12)	0.074	0.119	0.978
NG	2 (0.16)	11 (3.63)	0.04 (0.005–0.19)	<0.001	<0.001	0.053
UU	66 (5.19)	15 (4.95)	1.05 (0.58–2.01)	1.000	1.000	1.000
UP	317 (24.92)	27 (8.91)	3.39 (2.23–5.34)	<0.001	<0.001	<0.001
MH	75 (5.90)	8 (2.64)	2.30 (1.97–5.60)	0.021	0.057	0.841
MG	5 (0.39)	3 (1.00)	0.39 (0.07–2.56)	0.186	0.248	1.000
TV	19 (1.49)	4 (1.32)	1.13 (0.37–4.61)	1.000	1.000	1.000
Co-infections	87 (6.84)	12 (3.96)	1.78 (0.95–3.62)	0.066	0.119	0.946

SF specimens collected from female patients; *SM* Specimens collected from male patients; *CT Chlamydia trachomatis; NG Neisseria gonorrhoeae; UU Ureaplasma urealyticum; UP Ureaplasma parvum; MG Mycoplasma genitalium; MH Mycoplasma hominis; TV Trichomonas vaginalis; BH p value:* BH adjusted *p* value; $p_{100.000}$ *p* value after 100.000 random replicates

(Table 1). Prevalence of co-infections ranged from a minimum of 0.08% (1 sample) for NG DNA to a maximum of 5.19% (66 samples) for UP DNA. In particular, 18/23 (78.26%) CT DNA positive samples were detected in association with other microorganisms (Table 2). Moreover, despite the high prevalence of UP single infections, UP co-infections were 66/317 (20.82%). On the contrary, MH was mainly detected associated with other microorganisms (60/75, 80.00%) (Fig. 1).

Among the 303 samples collected from male patients (SM), 64 (21.12%) were positive for DNA for at least one microorganism with 52 (17.16%) samples positive for only one microorganism, 9 (2.97%) for two and 3 (1.00%) for three. In total, in 11 (3.63%) samples CT DNA was detected, in 11 (3.63%) NG DNA, in 15 (4.95%) UU DNA, in 27 (8.91%) UP DNA, in 8 (2.64%) MH DNA, in 3 (1.00%) MG DNA and in 4 TV DNA (1.32%), respectively. In 12 (3.96%) samples, a co-infection was detected (Table 1). Interesting, UP and NG co-infections were 11.11% (3/27 samples) and 9.09% (1/11), respectively. On the contrary, MH co-infections were 100.00% (8/8) (Table 2 and Fig. 1).

After 100.000 randomizations, statistically significant difference in prevalence between SF and SM was only detected for UP (24.92 vs 8.91%, $p_{100.000}$ <0.001, OR 3.39, 95% CI 2.23–5.34) (Table 1). On the other hand, no significant differences in prevalence of co-infections for each microorganism were observed (Table 2). Single and combinations of multiple infections detected are reported in Table 3. The most frequent combinations detected in SF were UP + MH (26, 2.04%), UU + MH (10, 0.79%) and UU + UP + MH (9, 0.71%), while UU + MH + TV (4, 1.32%), UU + MH (3, 0.99%) and UP + MH (2, 0.66%) were reported in SM, respectively.

Multivariate analysis on 100.000 random replicates showed that MH is associated with UU and UP (MH vs UU adjusted OR 15.84, 95% CI 8.42–29.86, MH vs UP adjusted OR 4.98, 95% CI 2.98–8.48) in SF, but it was not possible to reveal any significantly associated co-infection in SM (Table 4).

Discussion

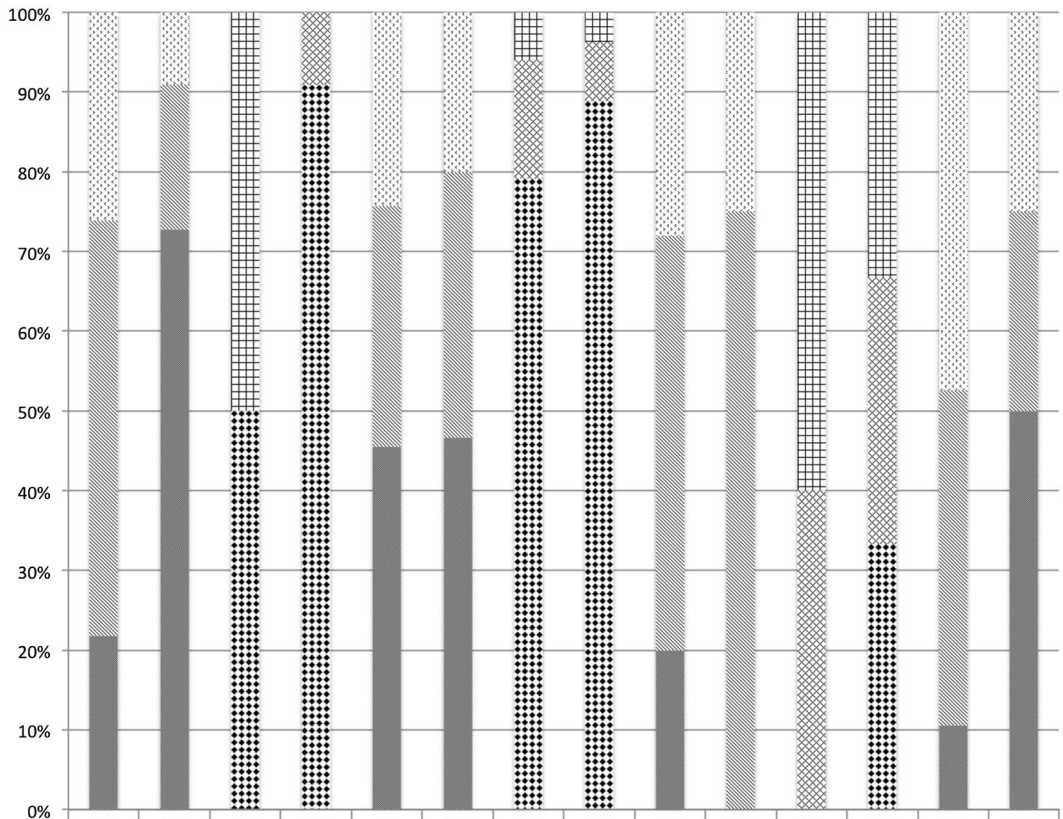
Urogenital tract infections are a major cause of morbidity in sexually active individuals all over the world. In particular, CT is the leading cause of bacterial STI and its most common clinical manifestations are mucopurulent cervicitis and urethritis in women, orchitis, epididymitis and testicular atrophy in men [17]. As the majority of infections are asymptomatic, so they are not recognized and treated, promoting a reservoir of infection and leading to pelvic inflammatory disease (PID) followed by subsequent infertility and ectopic pregnancy [18]. The prevalence of CT in healthy women has been estimated worldwide varying between countries and cities depending on prevailing environmental factors and population living habits. World Health Organization (WHO) reported a globally prevalence in women of 4.2%, with geographical values ranging from 1.8 to 7.6%, while in men a prevalence of 2.7%, with geographical values ranging from 1.3 to 5.2% [19]. Our data have shown a prevalence of 1.81% in female and 3.63% in male population, respectively.

NG is the etiological agent of gonorrhea, a significant health problem worldwide, especially in light of increasing resistance to currently used antibiotics [4]. The sequelae to untreated infections include PID, infertility, chronic pelvic pain and ectopic pregnancies. According to WHO data from 2005 to 2012, the global prevalence of *Neisseria gonorrhoeae* in women was 0.8% and the regional values accounted for 0.3 to 1.7%, while in men, the rate was 0.6% with regional values ranged from 0.3 to 1.0% [19]. Our

Table 2 Evaluation of co-infections in samples collected from female and male patients

Microorganisms	Co-infections, Nps/total (%, 95%CI) SF	Co-infections, Nps/total (%, 95%CI) SM	Odds ratio (95% CI) SF vs SM	*p* value	BH *p* value	$P_{100.000}$
CT	18/23 (78.26)	3/11 (27.27)	8.83 (1.46–72.66)	0.007	0.054	0.971
NG	1/2 (50.00)	1/11 (9.09)	7.42 (0.06–851.72)	0.295	0.438	1.000
UU	36/66 (54.55)	8/15 (53.33)	1.05 (0.29–3.75)	1.000	1.000	1.000
UP	66/317 (20.82)	3/27 (11.11)	2.10 (0.61–11.22)	0.318	0.438	1.000
MH	60/75 (80.00)	8/8 (100)	0.00 (0.00–2.66)	0.340	0.438	1000
MG	5/5 (100)	2/3 (66.67)	Inf (0.04-Inf)	0.375	0.438	1.000
TV	17/19 (89.47)	2/4 (50.00)	7.37 (0.36–167.89)	0.125	0.436	1.000

Nps/total number of positive samples/total number of samples positive for a specific microorganism; *SF* specimens collected from female patients; *SM* specimens collected from male patients; *CT Chlamydia trachomatis; NG Neisseria gonorrhoeae; UU Ureaplasma urealyticum; UP Ureaplasma parvum; MG Mycoplasma genitalium; MH Mycoplasma hominis; TV Trichomonas vaginalis; BH p value* BH adjusted *p* value; $P_{100.000}$ *p* value after 100.000 random replicates



	SF	SM	SF	SM	SF	SM	SF	SM	SF	SM	SF	SM	SF	SM
	CT		NG		UU		UP		MH		MG		TV	
Co-infection (with >1 microorganism)	6	1	1	0	16	3	19	1	21	2	3	1	9	1
Co-infection (with 1 microorganism)	12	2	0	1	20	5	47	2	39	6	2	1	8	1
Single infection	5	8	1	10	30	7	251	24	15	0	0	1	2	2

Fig. 1 Samples collected from female and male patients: evaluation of single infections, co-infections with another one microorganism and co-infections with more than one microorganism expressed as counts data (*table*) and percentage values (*bar plot*) calculated on the total of positive samples per microorganism. *SF* specimens collected from female patients; *SM* specimens collected from male patients; *CT* chlamydia trachomatis; *NG Neisseria gonorrhoeae; UU Ureaplasma urealyticum; UP Ureaplasma parvum; MG Mycoplasma genitalium; MH Mycoplasma hominis; TV Trichomonas vaginalis*. No significant differences between SF and SM were detected by Fisher's exact test on 3 × 2 matrices with BH's correction

results are in agreement with these findings with regard to women (0.16%), instead we have found slightly higher values in men (3.63%). Nevertheless, some studies conducted on sex workers in Bangladesh have shown a highest prevalence of NG with values ranging from 35.5 to 42% [20–22].

Also, genital mycoplasma and ureaplasma are suspected of contributing to a number of pathological conditions such as NGU, preterm birth, perinatal morbidity and mortality [23]. Nevertheless, because of their colonizing role, it is difficult to clarify the pathogenic involvement of these microorganisms suggesting that epidemiological studies with healthy people and patients with urogenital disorders are needed. Previously, because of the absence of the mRT-PCR techniques, many reports had suggested the role of Ureaplasma without discriminating between UU and UP [24]. A study of Yamazaki et al. (2012) by PCR detection reported that the prevalence of UP and UU was 41.7 and 8.9%, respectively [25]. Other studies performed on healthy women reported UP rate about 50% while UU rate about 10% [24]. Our data confirm the high UP prevalence in the analyzed population. In particular, we have recovered UP DNA in 24.92% woman and in 8.91% men.

Among mycoplasmas, MG and MH are involved in urogenital tract infections. Increasing evidence supports a significant role for these in the pathogenesis of chorioamnionitis, premature membrane rupture and preterm labor in pregnant woman [26]. In a Swedish study, MG accounted for 6.3% in women and 6.0% in men. In the same study, MG was found more often than CT both in women and men [27]. On the other hand, Bujold et al. (2008) have published data with a major rate of prevalence of MG in women (9.5%) and men (10.6%) [28]. In Brazil, Rodrigues

Table 3 Single and multiple infections detected in SF and SM

SF: single and multiple infections	Number (%)	SM: single and multiple infections	Number (%)
CT	5 (0.39)	CT	8 (2.64)
NG	1 (0.08)	NG	10 (3.30)
UU	30 (2.36)	UU	7 (2.31)
UP	251 (19.73)	UP	24 (7.92)
MH	15 (1.18)	–	–
–	–	MG	1 (0.33)
TV	2 (0.16)	TV	2 (0.66)
CT + UU	4 (0.31)	–	–
CT + UP	6 (0.47)	–	–
CT + MH	2 (0.16)	CT + MH	1 (0.33)
–	–	CT + MG	1 (0.33)
–	–	NG + UU	1 (0.33)
UU + UP	6 (0.47)	–	–
UU + MH	10 (0.79)	UU + MH	3 (0.99)
–	–	UU + TV	1 (0.33)
UP + MH	26 (2.04)	UP + MH	2 (0.66)
UP + MG	2 (0.16)	–	–
UP + TV	7 (0.55)	–	–
MH + TV	1 (0.08)	–	–
CT + NG + UP	1 (0.08)	–	–
–	–	CT + UU + MG	1 (0.33)
CT + UP + MH	2 (0.16)	–	–
UU + UP + MH	9 (0.71)	UU + UP + MH	1 (0.33)
UU + MH + TV	3 (0.24)	UU + MH + TV	4 (1.32)
UP + MH + TV	2 (0.16)	–	–
UP + MG + TV	1 (0.08)	–	–
CT + UU + UP + MG	1 (0.08)	–	–
CT + UU + MH + MG	1 (0.08)	–	–
CT + UP + MH + TV	1 (0.08)	–	–
UU + UP + MH + TV	1 (0.08)	–	–
UU + UP + MH + MG + TV	1 (0.08)	–	–

SF specimens collected from female patients; *SM* specimens collected from male patients; *CT Chlamydia trachomatis; NG Neisseria gonorrhoeae; UU Ureaplasma urealyticum; UP Ureaplasma parvum; MG Mycoplasma genitalium; MH Mycoplasma hominis; TV Trichomonas vaginalis*

et al. (2011) reported a frequency of 0.9% in women [29]. In our study, similar results were obtained in women. In a USA study, MH was detected in 21–53% of asymptomatic and sexually active women, and its prevalence was slightly lower in men [30]. A recent survey performed on recovered women in health clinic in Australia reported the rate of colonization of 13.7% [31]. Our data have shown a rate of MH DNA positive of 5.90 and 2.64% in female and male population, respectively.

TV is the most prevalent protozoan agent involved in the STIs. Its global prevalence has been estimated at 8.1% for women and 1.0% for men [32]. Newman et al. (2015) reported a globally prevalence in women of 5.0% with geographical data ranging from 1.0 to 11.5%, while in men a prevalence of 0.6% with geographical values ranging from 0.1 to 1.3% [19]. We have found prevalence values of 1.49% among women and 1.32% among males.

In our study, we have also evaluated the co-infections by different pathogens. Our data suggest an association between MH and UU/UP infection in SF. Interestingly, this finding was not confirmed in SM, thus suggesting the presence of different patterns of co-infections among females and males. Some studies report that UU and MH infections are associated with vaginitis, cervicitis and pelvic inflammatory disease in women [33]. Other studies indicate that they are also associated with adverse pregnancy outcomes despite there are some controversies for the presence of these bacteria as commensal in vaginal flora [34]. In a study of Kwak et al. (2014) patients infected by UU and MH have shown a significant

Table 4 Evaluation of association among different microorganisms by univariate (Fisher's exact test BH corrected p values and after 100.000 random replicates) and multivariate (logistic regression models for each microorganism evaluated on 100.000 random replicates) analysis

Association of co-infections in SF							
	CT	NG	UU	UP	MH	MG	TV
Fisher's test and BH correction							
NG	0.057						
UU	0.003	1.000					
UP	0.032	0.573	0.817				
MH	<0.001	1.000	<0.001	<0.001			
MG	0.131	1.000	0.046	0.032	0.053		
TV	0.413	1.000	0.006	0.001	<0.001	0.006	
p values on 100.000 replicates							
NG	0.838						
UU	0.242	1.000					
UP	0.718	1.000	1.000				
MH	0.137	1.000	<0.001	<0.001			
MG	0.998	1.000	0.608	0.658	0.692		
TV	1.000	1.000	0.368	0.057	0.013	0.501	
Logistic regression models, *p* values on 100.000 replicates							
CT		0.462	0.698	0.934	0.520	0.331	1.000
NG	0.462		1.000	1.000	1.000	1.000	1.000
UU	0.691	1.000		0.811	<0.001	1.000	0.993
UP	0.933	1.000	0.884		<0.001	1.000	0.541
MH	0.735	1.000	<0.001	<0.001		1.000	0.164
MG	0.992	1.000	1.000	1.000	1.000		0.438
TV	1.000	1.000	0.998	0.563	0.101	0.626	
Association of co-infections in SM							
Fisher's test and BH correction							
NG	1.000						
UU	1.000	1.000					
UP	1.000	1.000	1.000				
MH	0.776	1.000	<0.001	0.138			
MG	0.037	1.000	0.496	1.000	1.000		
TV	1.000	1.000	0.091	1.000	0.428	1.000	
p values on 100.000 replicates							
NG	1.000						
UU	1.000	1.000					
UP	1.000	1.000	1.000				
MH	1.000	1.000	0.154	0.969			
MG	0.598	1.000	1.000	1.000	1.000		
TV	1.000	1.000	0.905	1.000	1.000	1.000	
Logistic regression models, *p* values on 100.000 replicates							
CT		1.000	1.000	1.000	1.000	0.623	1.000
NG	1.000		0.996	1.000	1.000	1.000	1.000
UU	1.000	1.000		1.000	1.000	1.000	0.862
UP	1.000	1.000	1.000		1.000	1.000	1.000
MH	0.981	1.000	0.669	1.000		1.000	1.000
MG	0.616	1.000	0.835	1.000	1.000		1.000
TV	1.000	1.000	0.768	1.000	1.000	1.000	

CT Chlamydia trachomatis; NG Neisseria gonorrhoeae; UU Ureaplasma urealyticum; UP Ureaplasma parvum; MG Mycoplasma genitalium; MH Mycoplasma hominis; TV Trichomonas vaginalis

reduction of gestational age at birth and birth weight while incidence of preterm birth, admission in neonatal intensive care unit and chorioamnionitis were significantly increased when compared to patients positive for UU alone [35]. Moreover, some data suggest that co-infection UU-MH may be associated with an increased drug resistance [33]. In our study, MH single infection was only detected in 20.00% of SF and in 0.00% of SM suggesting that other factors may be needed to contribute to MH infection, as yet suggested by Zhu et al. (2012) [33, 36]. Finally, although in their study on preterm birth Kataoka et al. (2006) reported co-infections by MH and UP (10.7%) their role as pathogens should be further investigated [37].

There are some limitations of this study. First, the reported data are not representative of overall Apulia population so prevalence values are not generalizable. Second, viruses and other bacterial pathogens sexually transmitted were not investigated. Third, clinical data were not available to assess risk factors connected with the presence of specific pathogens and the presence/absence of clinical signs and symptoms. Moreover, after a previous report regarding the association between CT and UP in healthy women attending the first prenatal visit, Yamazaki et al. (2014) reported a higher CT growth in the presence of UP and interferon-γ [38]. However, such association was not confirmed in our study. Our data, finally, suggest the presence of some co-infectious associations among the seven analyzed pathogens that may vary between men and women. In the end, multiplex RT-PCR assay for sexually pathogens detection in our study was extensively analyzed by some authors with promising results showing sensitivities from 93.94 to 100% and specificities from 96.55 to 100%, respectively [14, 39, 40].

Despite the abundance of reports on those microorganisms, works linking the microorganisms to clinical conditions progress slowly, mainly due to the fastidiousness and time-consuming of culture methods [6]. Nevertheless, it is important to increase either the vigilance or the quick and accurate identification of responsible agents for STIs to reduce the sequelae or the complications on reproductive organs.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

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