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PROTOZOOLOGY - ORIGINAL PAPER

Trichomoniasis in a tertiary hospital of Madrid, Spain (2013–2017): prevalence and pregnancy rate, coinfections, metronidazole resistance, and endosymbiosis

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Abstract

Trichomoniasis is the most prevalent curable sexually transmitted infection (STI) worldwide and a risk factor for the acquisition of other STIs and adverse pregnancy outcomes. The objectives of this study were to determine the prevalence of *T. vaginalis* and related coinfections in women attending a third-level hospital of Madrid (Spain). A retrospective study of 24,173 vaginal exudates from women with suspected vaginitis was conducted between 2013 and 2017. Likewise, among *T. vaginalis* positive samples, co-occurrence with gonorrhea, chlamydia, syphilis, VIH, *Mycoplasma hominis*, and *Ureaplasma urealyticum* was checked. Moreover, seven *T. vaginalis* isolates from 2017 were randomly collected for endobionts, drug resistance, and microsatellite (MS) instability determinations. The prevalence of *T. vaginalis* was 0.8% between 2013 and 2017. Less than 20% of patients with trichomoniasis were submitted to a complete screening for other genital pathogens. From that, two patients were coinfected with chlamydia and three with syphilis. Surprisingly, 6.4% of positive samples were diagnosed among pregnant women, showing an alarming increase from 3.2% (2014) to 10% (2017). Among the isolates randomly analyzed, five carried *T. vaginalis* virus, five harbored mycoplasmas, and one was metronidazole-resistant. The molecular genotyping showed a high variability in the three MS evaluated. To our knowledge, this is the first study in Spain that evaluates the prevalence of trichomoniasis in general and pregnant population and includes biomolecular determinations. These results warn about the increasing prevalence and highlight the importance of including *T. vaginalis* detection in routine gynecological revisions with special emphasis on childbearing age women and patients with previous STIs.

Keywords *Trichomonas vaginalis* · Prevalence · Coinfections · Pregnancy · Symbiosis · Metronidazole resistance

Introduction

Human trichomoniasis is the most prevalent nonviral sexually transmitted infection (STI) in the world. The annual number of *Trichomonas vaginalis* infections (143 million) is higher than those caused by *Chlamydia trachomatis* (131 million), *Neisseria gonorrhoeae* (78 million), and *Treponema pallidum* (5.6 million) (Rowley et al. 2019). Even so, it is considered as one of the 5 Neglected Parasitic Infections by CDC (2020). This is due to the fact that trichomoniasis is not a notifiable disease and also that *T. vaginalis* can parasite without causing symptoms, especially in men that become asymptomatic carriers, while in women, the clinical profile varies from asymptomatic patients in almost half of the cases (Petrin et al. 1998) to severe inflammatory and invasive processes along the genitourinary tract that can lead in a neoplastic process (Viikki et al. 2000; Yang et al. 2018).

In this neglected scenario, the aforementioned explains the scarce epidemiological reports that have been conducted. In Spain, only two studies were carried out in Granada (Carrillo-Ávila et al. 2017; Sorlózano-Puerto et al. 2018). Fortunately, in the last years, the interest of these pathologies has risen

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substantially owing to the increase burden of STI cases (Rowley et al. 2019).

Altogether with the important epidemiological aspect, *T. vaginalis* is a proven factor capable of increasing up to 2.7 times the risk of acquiring HIV (Van Der Pol et al. 2008) and has been associated with a higher prevalence of other urogenital pathogens, such as *Chlamydia trachomatis Herpes simplex* type 2 (HSV-2) *Treponema pallidum*, *Neisseria gonorrhoeae* (Helms et al. 2008; Allsworth et al. 2009), and human papillomavirus, especially the high-risk serotype 16 (HPV-16) (Lazenby et al. 2014).

Besides, the only two drugs approved by the FDA and cited by the European protocol guidelines for trichomoniasis treatment are metronidazole and tinidazole (Meites et al. 2015). There are no alternative treatments as effective as these 5 - nitroimidazoles. It is important to highlight that the number of refractory cases of trichomoniasis range near 10% (Kissinger 2015) and protocols recommend repeating the treatment or increasing the drug dosage. Despite all of these, the number of studies related to resistant *T. vaginalis* in the population is low, with no data referring to Spain.

It is noteworthy that in the last years, diverse researchers have focused their studies in the ability of *T. vaginalis* to harbor endosymbionts and its potential implication in the pathogenesis of the flagellate (Fichorova et al. 2017). In light of these findings, it would be of interest to study in depth the possible relationship between metronidazole resistance and the presence of endosymbionts. One of the best-known endosymbionts is *Mycoplasma hominis* (Dessì et al. 2005), a microorganism often isolated in the genitourinary tract, which can cause symptomatic infections with serious consequences in pregnant women (Murtha and Edwards 2014; Thi Trung Thu et al. 2018). Nevertheless, the consequences of this bacterial-parasite association are still unknown. Whereas some studies seem to show a correlation between the presence of *M. hominis* and a greater resistance to metronidazole by *T. vaginalis* (Margarita et al. 2016; Fürnkranz et al. 2018), others present contradictory results (Xiao et al. 2008; da Luz Becker et al. 2015). On the other hand, recent bibliographical references have associated *T. vaginalis* harboring *Mycoplasma genitalium*, a currently emerging specie intimately ligated with premature births (Costello et al. 2013). Although more studies are necessary, these microorganisms might influence the pathogenesis of *T. vaginalis* and, therefore, facilitate its transmission and hinder its treatment.

Collectively, and in an attempt to shed more light on the inexistent epidemiological and resistance studies of this STI in Spain, the major goal of this work was to execute a retrospective study of patients diagnosed of trichomoniasis in a Spanish tertiary hospital. The prevalence, potential risk factors, and coinfections with other STI were evaluated. The resistant cases to metronidazole, the presence of endosymbionts, and the study of microsatellites (MS) to analyze the genetic diversity of *T. vaginalis* isolates were also executed in clinical samples.

Materials and methods

Study setting

A retrospective study was conducted between January 2013 and December 2017 in Puerta de Hierro Majadahonda Hospital (Northwest Madrid, Spain), which covers a population of about 550,000 people. During that period of time, a total of 24,173 vaginal exudates (17,265 women) were processed for the presence of *T. vaginalis* as well as for the coinfection with *N. gonorrhoeae, C. trachomatis, T. pallidum*, and HIV. In addition, *M. hominis* and *U. urealyticum* were also tested. Exclusion criteria were not applied, and pregnant women received special attention. Because of the isolation of *T. vaginalis* in males turned to be scarce, they were not included in the study. Likewise, seven positive samples were randomly tested for metronidazol resistance, intracellular presence of endosymbionts (*Trichomonas vaginalis* virus [TVV] and mycoplasmas), and microsatellite genotyping.

Sample collection

Vaginal exudate samples were collected in a sterile vaginal swab and placed into a sterile sampling container with Stuart medium (Deltalab Amies, Spain) for delivery to the laboratory for microbiological culture and species identification.

Microbial identification

All samples were tested upon their reception in the laboratory. *Trichomonas* spp. was isolated by microscope examination and selected swabs were seeded in Roiron culture medium (MAIM, Spain) following standard protocols of the Spanish Society of Infectious Diseases and Clinic Microbiology, SEIMC (Galán Montemayor et al. 2018). *N. gonorrhoeae* and *C. trachomatis* were detected in female endocervical swabs (BD Universal Viral Transport (UVT)) by Real Time PCR (BDMax, Becton Dickinson, USA) according to the manufacturer's instructions. Additionally, *T. pallidum* and HIV were diagnosed in serum by automated chemiluminescence immunoassay using the Liaison®X (DiaSorin, Italy) and ADVIA Centaur®XP Immunoassay System (Siemens, USA). *M. hominis* and *U. urealyticum* were detected by the IST2 colorimetry assay (Biomérieux, France). Infection was considered over colonization when ≥ 10.000 CCU (Colour Changing Units) were detected, following manufacturer's instructions.

Trichomonas vaginalis culture

T. vaginalis isolates were cultivated at 37 °C and 5% CO_2 in modified trypticase-yeast extract-maltose (TYM) medium supplemented with 10% (v/v) heat-inactivated fetal bovine

serum (FBS) and antibiotic solutions (100 UI/ml penicillin, 100 μl/ml streptomycin, and 100 μl/ml gentamicin). Cultures were passaged every 1–2 days into fresh medium until axenic cultures were obtained. The biological and molecular characterizations were executed using clinical isolates with less than 8 passages to conserve their wild-type properties.

Metronidazole susceptibility assay

The susceptibility to metronidazole was performed following the protocol described by Vanácová et al. (2007) with slight modifications. Briefly, 10^5 trichomonads/100 μl were added to each well in a 96-well microtiter plate. Then, they were incubated at 37 °C 5% CO_2 with serial twofold dilution of metronidazole (256 to 2 μg/ml) for 48 h. The minimum lethal concentration (MLC) was defined as the lowest solution of drug in which no motile trophozoites are observed in the inverted microscope. All experiments were carried out in duplicate. Low drug resistance was defined as a MLC from 50 to 200 μg/ml after 48 h of incubation, being highly resistant with MLC > 200 μg/ml (Narcisi and Secor 1996).

DNA extraction and amplification

Total nucleic acids were extracted from in vitro cultures after at least three passages with gentamicin to assure the elimination of possible mycoplasmas in the supernatant. An aliquot of each isolate was centrifuged at 300×*g* 3 min. Then, the pellet was digested with 1.4 μL of PCR buffer at 90 °C for 10 min, and after that, 1.1 μL of proteinase K (20 mg/ml; Sigma-Aldrich) was added and incubated for 3 h at 65 °C following the protocol of Garcia-Sánchez et al. (2009). The correct extraction of *T. vaginalis* DNA from the different samples was confirmed by PCR using the forward primer 18SF 5′-ACG CCG TAG TCT GAA TTG GC-3′ and the reverse primer 18SR 5′-AGA CAG GTC AAC CCA CGC AC-3′ designed by Ibáñez-Escribano et al. (2014) for the amplification of a conserved amplicon of the 18S rRNA region.

The PCR reactions were carried out using the PureTaq Ready-to-Go kit (GE Healthcare, UK) following the manufacturer's recommendations in a final volume of 25 μL. PCR amplicons were visualized by electrophoresis in 1.5% agarose gel and stained with GelRed® Nucleic Acid (Biotium-BIOGEN Científica, Spain).

Determination of microsatellites polymorphism

The three most polymorphic microsatellites (MS06, MS129, and MS184) from the panel characterized by Conrad et al. (2011) were selected for the characterization of the isolates. The primers and PCR reactions were executed following Conrad et al. (2011) protocol. The amplicons length was analyzed by the Genomic Unit of the Research Support Centre (Complutense University of Madrid). Sequencing results were analyzed using Peak Scanner Software v1.0.

Mycoplasma endobionts identification

The presence of mycoplasmas inside the trophozoites were analyzed using Mycoplasma Detection Kit (Southern Biotech, Birmingham, USA), which amplifies the 16S rRNA of 19 different species from genera *Mycoplasma*, *Ureaplasma*, and *Acholeplasma*.

TVV determination

TVV presence was evaluated by indirect fluorescence, using a monoclonal antibody (MoAb) for the detection of the immunogenic parasitic protein P270 on the surface of the trophozoites. This protein is located in the cytoplasmic body of *T. vaginalis*; however, when the parasite harbors TVV, P270 is expressed on its surface. The isolates were fixed in a 2% formaldehyde solution at 4 °C. Then, 25,000 fixed trophozoites were added in acetone-resistant spots (Biomérieux, France) and incubated in acetone 100% at 4 °C for 10 min. Slides where incubated with a 1:100 diluted anti-P270 MoAb (C20A3) for 1 h at 37 °C (Alderete and Kasmala 1986). After washing three times with PBS-2% Tween 80, the samples were incubated again 1 h at 37 °C with the secondary antimouse IgG-FITC (1:200). Finally, and after washing the slides with PBS-Tween 80 2%, samples were stained with Evan's Blue for 5 min and fixed with Fluoprep ® prior to be visualized in an Olympus BH2 fluorescence microscope.

Statistical analysis

Medical records, from the vaginal exudates where *T. vaginalis* was isolated in this period of time (2013–2017), were screened. A retrospective and descriptive study was done. Statistical analysis was performed using chi-square test (SPSS, v.25, IBM). A $p < 0.05$ was considered significant.

Ethical statement

The study protocol was carried out according to the Declaration of Helsinki. The hospital's ethics committee "Comité Ético de Investigación con Medicamentos del Hospital Universitario Puerta de Hierro Majadahonda-Madrid (CEIm)" granted permission (Acta n° 21.17) to access to medical records and use them for this study. Data analysis were performed using an anonymous database.

Results

Epidemiological study

A total of 138 patients were positive for *T. vaginalis* from a whole sample of 17,265 women during this period of time (2013–2017) with a total prevalence of 0.8%. Two patients had a recurrence event with 1 year difference between the two episodes. The mean age was 38.8 ± 10.9 years old among global female population. Figure 1 shows the monthly prevalence trend during this 5-year period, with two peaks: from February to April and then in September. No statistical differences were found in the seasonal distribution of trichomoniasis cases ($p < 0.05$). Along these 5 years, prevalence decreased gradually until 2016, when the falling trend was broken, and the number of infections diagnosed was almost duplicated (Fig. 2). Half of patients were Mediterraneans (51.4%), followed by South and Central Americans (31.9%), East Europeans (11.6%), North Africans (4.3%), and other nationalities (1.4%).

Among the 138 female patients infected with *T. vaginalis*, only 54.3% presented a description about the symptomatology in their medical records. From these 75 patients, only the 9.2% were asymptomatic. The most frequent symptoms were leukorrhea (69.6%) and pruritus (52.2%), usually simultaneously, while others referred vaginitis (11.6%), dysuria (5.8%), and erythema (7.2%).

The inflammation degree was determined by the amount of leukocytes observed at the microscope. From the 138 patients, more than half of the patients exhibit few (30%) or no leukocytes (28%), while the rest of the samples (42%) presented leukocytosis. In this sense, it must be highlighted that an altered flora was detected in 66.4% of patients, followed by usual flora (21.4%) and candidiasis (11.4%) (Table 1). Moreover, twelve patients (8.6%) were attended by Urgent Care Services, of whom two were pregnant and other two patients were coinfected with *U. urealyticum*.

Regarding these 12 patients, the coinfection with other STI pathogens was unknown as in most of the cases screening was not requested. All of them presented intense leukorrhea and pruritus, pain, or erythema, and in one of them also light bleeding not caused by menstruation. Also, half of them presented leukocytosis. Finally, one patient developed cervical cancer 2 years after *T. vaginalis* diagnosis.

In this context, we searched for those patients that, after *T. vaginalis* diagnosis, were also requested for coinfection screening with *C. trachomatis* (26), *N. gonorrhoeae* (139), HIV (38), *T. pallidum* (34), *M. hominis* (29), and *U. urealyticum* (29) (Table 1). No coinfections were found in relation with *N. gonorrhoeae* or HIV, whereas 2 cases of *C. trachomatis* and 3 cases of *T. pallidum* were detected. Besides, *M. hominis* and *U. urealyticum* happened to be isolated in 12 and 17 patients, respectively (Table 1).

Prevalence of *T. vaginalis* infection among pregnant population represented the 6.4% in this study. Comparing with nonpregnant women, the mean age was 11 years lower (28.6 ± 7.2 vs. 39.6 ± 10.9). Half of this group were South Americans (55.5%), followed by Mediterraneans (33.3%) and Eastern Europeans (11.1%). According to symptoms, pregnant women were more frequently asymptomatic than nonpregnant (30% vs. 2.3%) and also laboratory results indicate that 77.7% of vaginal exudates from pregnant population exhibited low or no leukocytes. Moreover, the most common symptoms in this population were leukorrhea (40%) and upper quadrant pain (30%).

Biomolecular characterization

Seven over the thirty *T. vaginalis* positive samples isolated at the hospital during 2017 were randomly selected for biomolecular characterization. One low-resistant isolate was found (PH239) with a MLC = 64 μg/ml. Curiously, this patient returned to the hospital after treatment due to the persistence of symptoms. The rest of the isolates showed minimum lethal concentration (MLC) lower than 16 μg/ml after 48 h.

Regarding endobionts, 71.4% of the samples carried mycoplasmas or TVV. The resistant isolate (PH239) harbored mycoplasmas but was TVV-negative. The asymptomatic patient (PH351) was only positive for TVV as shown in Table 2.

The molecular genotyping showed a high variability in the MS lengths when comparing the different isolates (Table 2). At least three different alleles were detected for each MS, and no pattern was identified when comparing molecular data.

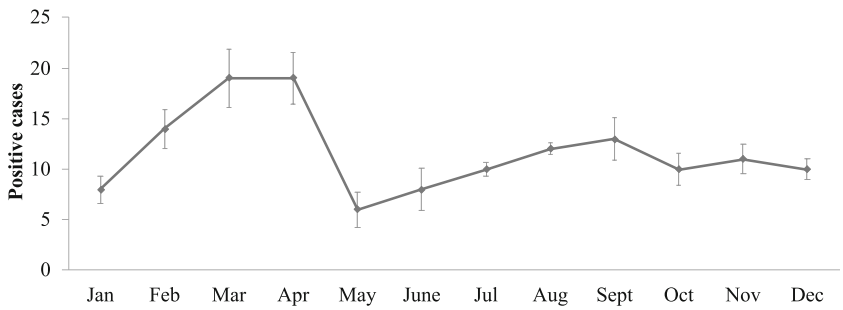

Fig. 1 Case distribution of *Trichomonas vaginalis* positive samples per month

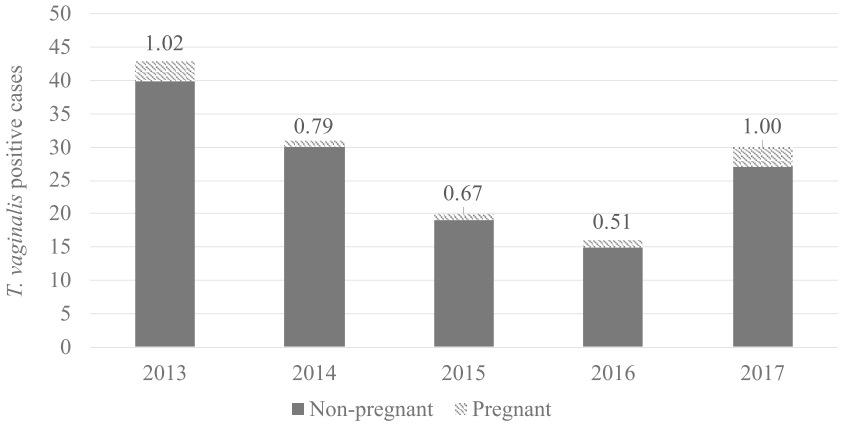

Fig. 2 Global prevalence rate of *Trichomonas vaginalis* per year and positive cases in nonpregnant and pregnant women

Discussion

The real burden of trichomoniasis is unknown. Although this parasitic infection is the most prevalent curable STI (Rowley et al. 2019), is still being characterized as a non-notifiable disease. Moreover, the WHO in the recent "Global Health Sector Strategy on STIs 2016-2021: towards ending STIs" pays special attention to reduce syphilis and gonorrhea infections and targets HPV vaccination, but no specific strategies are proposed to reduce trichomoniasis prevalence (WHO 2016). Despite the high prevalence, many aspects related with its pathobiology, as the diverse clinical profile (Kissinger 2015), the consequences of harboring bacteria and viruses (Fichorova et al. 2017), the risk of developing certain neoplasias (Viikki et al. 2000; Sutcliffe et al. 2012), or the complex genome (Conrad et al. 2013), are unresolved questions that justifies why trichomoniasis should deserve more attention.

In this study, 140 samples (0.8%) were positive for *T. vaginalis* among a total number of 23,173 vaginal exudates from women attending to the medical centers and gynecologists assisted by a third-level hospital during a period of time of 5 years (2013–2017). Our results are in consonance with the report of Sorlózano-Puerto et al. (2018) that diagnosed *T. vaginalis* in the 1% of the population screened and with Carrillo-Ávila et al. (2017), which reported a prevalence of 2.4% *T. vaginalis* positive women between 2011 and 2014 in other third-level hospital of the South of Spain, after the analysis of 5230 samples. Furthermore, monthly prevalence distribution was almost identically in both studies, suggesting that the epidemiology in this country is related to cultural behavior, as the two peaks were after Christmas and Summer Holidays with significant differences when comparing the number of *T. vaginalis* cases per month (Fig. 1). Although the prevalence detected in this study is slightly lower than the reported in the aforementioned research, the different methods used could have influenced the prevalence rates. Even though PCR techniques used by Carrillo-Ávila et al. (2017) are more sensitive and less subjective (Khatoon et al. 2015), the wet-mount microscopic observation and *T. vaginalis* culture are still the "gold-standard" methods implemented in the main hospitals and clinics for trichomoniasis diagnosis.

This report corroborates that the epidemiological burden of trichomoniasis varies greatly throughout the world. The dearth of multicenter studies executed by public health systems difficult the exact estimation of trichomoniasis. Consequently, the wide range of *T. vaginalis* rates depends on the method used for diagnosis, the population studied or the sample size. For example, in USA, depending on the study consulted, trichomoniasis ranges from 2.5 to 26.2% (Meites et al. 2013), while in Asia, some studies detected 3.1% in South Korea (Goo et al. 2016), but others reported 10.4% (Ryu and Min 2006). Likewise, in the African continent, depending on the region, the prevalence in Ghana can fluctuate from 2.7% (Apea-Kubi et al. 2005) to 18.1% (Squire et al. 2019).

An important aspect derived from this cross-sectional study is the alarming increase of trichomoniasis cases during pregnancy. From the total cases of trichomoniasis detected on vaginal swabs during this period (2013–2017), 6.4% were diagnosed among pregnant women. When analyzing the results in detail, it should be noted that since 2014, trichomoniasis has tripled from 3.2% in 2014 to 10% in 2017 in this specific population. This prevalence was similar to the rates detected

Table 1 Number of samples screened for other pathogens among the 140 *T. vaginalis* positive cases

	No. (%) of cases analyzed	Positives
Chlamydia trachomatis	26 (18.6%)	2
Neisseria gonorrhoeae	139 (99.3%)	0
HIV	38 (27.1%)	0
Treponema pallidum	34 (24.3%)	3
Mycoplasma hominis	29 (21.1%)	12
Ureaplasma urealyticum	29 (21.1%)	17

Table 2 Clinical and biomolecular results of seven *Trichomonas vaginalis* isolates randomly selected

	Biomolecular characterization			Molecular characterization: MS length (bp)			Clinical data				
	TVV	Mycoplasmas	MLC (μg/ml)	MS06	MS129	MS184	Age	Leukocytes*	Vaginal flora	Symptoms	Pregnancy
PH814	+	–	8	405	185	247	54	+	Normal	Pruritus	No
PH913	+	+	16	395	186	244	56	++	Normal	Leukorrhea	No
PH751	–	+	16	408	180	244	20	–	Candidiasis	Hypogastric pain, leukorrhea	Yes
PH351	+	–	8	396	183	250	54	++	Candidiasis	Asymptomatic	No
PH239	–	+	64	408	179	250	55	++	Altered	Pruritus, pain, leukorrhea	No
PH467	+	+	8	395	183	250	42	–	Altered	Unknown	No
PH637	+	+	8	408	185	250	46	++	Altered	Pruritus, leukorrhea	No

*Presence of leukocytes: absence (−); few leukocytes (+); frequent/high leukocytes (++)

in the Brazilian pregnant population (dos A Gatti et al. 2017). Furthermore, among the pregnant women diagnosed during these 5 years, one patient suffered a spontaneous abortion, whereas another had a premature rupture of membranes, causing a preterm birth at the 35th week of pregnancy. It is important to highlight that this patient was also diagnosed with *U. urealitycum*. In this line, taking into account that *M. hominis* and *U. urealyticum* also increase the risk of adverse pregnancy outcomes (Germain et al. 1994; Capoccia et al. 2013), it would be highly recommended screening for coinfections when *T. vaginalis* is detected, at least in pregnant women. These data collectively reinforce other studies that evidence the association between trichomoniasis and an increased risk of adverse perinatal morbidity (Silver et al. 2014) and reaffirm the need for screening trichomoniasis and other genitourinary pathogens in pregnant population in order to avoid evitable consequences during pregnancy and labor.

Regarding inflammation, over half of the analyzed cases in this research presented few or non-leukocytes, though most of the patients presented an altered vaginal flora. Therefore, *T. vaginalis* would promote a light inflammatory pathology and vaginal dysbiosis owing that *T. vaginalis* can phagocyte protecting bacterial microbiota (Margarita et al. 2016). Thus, the fact that the majority of symptoms were not severe (leukorrhea, pruritus) could explain why symptoms were not considered significant neither by the patients nor by the clinicians. As a consequence, this disease is often underdiagnosed and undervalued.

T. vaginalis is a key player in the risk of acquiring other genitourinary pathologies like HIV, human papillomavirus, herpes simplex virus, *N. gonorrhoeae, C. trachomatis*, and *T. pallidum* (Van Der Pol et al. 2008; Allsworth et al. 2009; Lazenby et al. 2014). Considering that these STI share the same mode of transmission, it was alarming that a high percentage of patients, between 72.5 and 81.2% depending on the pathogen, were not subjected to a large STI screening after a positive diagnosis of trichomoniasis, not even in pregnant women (Table 1). According to this, it is quite relevant that only *N. gonorrhoeae* was evaluated in more than 99% vaginal samples. Nevertheless, this was a consequence of how vaginal exudates are processed at our Microbiology lab. Almost all vaginal swabs come along with their endocervical swab, which are seeded in Martin-Lewis Medium, independently of clinician's requests. Consequently, the real impact of trichomoniasis as a marker of sexual behavior and potential inductor of other STI cannot be evaluated. Unfortunately, taken together these prevalence data in general and pregnant population and the lack of secondary STI determinations, our findings are in consonance with the fact that trichomoniasis is a neglected infection (Meites et al. 2013) that receives insufficient attention by the public health systems.

Likewise, the intriguing endobiont association between *T. vaginalis* and mycoplasmas, as well as certain viruses belonging to the Totiviridae family, has gained interest to different research groups in the last years (Margarita et al. 2016; Graves et al. 2019). The published prevalence of mycoplasma-positive trichomonads ranged from 20 to 90% (Butler et al. 2010; Dessì et al. 2005), while *T. vaginalis* harboring TVV also varies from 13.6 to 100% (Graves et al. 2019). It is worth pointing out that a high percentage of the isolates studied (71.4%) harbored bacteria or viruses, and in 42.9% of them, both endobionts were present in the same sample (Table 2). The remarkable high association between these organisms can be explained by the fact that, inside *T. vaginalis* trophozoites, *M. hominis* is protected from drug therapy as well as it is able to replicate (Vancini and Benchimol 2008). Not only that but also it is transmitted to other mycoplasmas free-trichomonads or vaginal epithelial cells (VECs) (Rappelli et al. 2001) using *T. vaginalis* as a "Trojan horse" (Dessì et al. 2005). On the other hand, infected trophozoites exhibit a more evident amoeboid transformation

and become more virulent (Vancini et al. 2008). Moreover, a recent study demonstrated that *M. hominis* inside the parasite cause a downregulation of metronidazole susceptibility-associated genes, contributing to *T. vaginalis* drug resistance (Fürnkranz et al. 2018). Interestingly, we noted that one of the five *T. vaginalis* mycoplasmas–positive samples was metronidazole-resistant, whereas the two mycoplasma-negative isolates exhibit the lowest metronidazole-MIC concentrations (Table 2).

Both *M. hominis* and *T. vaginalis* pathogens have been separately linked to adverse pregnancy outcomes as intrauterine growth retardation, preterm birth, and perinatal mortality or morbidity (Germain et al. 1994; Capoccia et al. 2013). Also, TVV modulates the expression of *T. vaginalis* proteins (CPs) (Provenzano et al. 1997) and, as a consequence, the immunopathology of the parasite. It is important to highlight that some studies have shown metronidazole treatment during pregnancy causes the death of trichomonads and so the liberation of internal microorganisms, allowing free *M. hominis* to infect vaginal epithelial cells (Thi Trung Thu et al. 2018), whereas the release of TVV induces proinflammatory signals (Fichorova et al. 2012). In both cases, the inflammatory response triggered by the immune system to these endobionts could provoke sequelae during pregnancy as well as promote susceptibility to HIV infection. Due to the importance of providing patients with the safest and most effective treatment, especially during pregnancy, more studies relating this symbiosis to pregnancy outcomes are needed.

In the last decades, different attempts to genotype *T. vaginalis* using RAPD (Vanácová et al. 2007), RFLP (Zhang et al. 2018), and ITS (Ibáñez-Escribano et al. 2014; Ertabaklar et al. 2018) have proved the high molecular polymorphism of this protozoan. The evaluation of the three more polymorphic microsatellites proposed by Conrad et al. (2011), in the seven clinical isolates, has also exhibited a remarkable variability with at least three alleles in each MS. Although the number of samples is not enough to affirm the usefulness of these markers, our observations agree with the observations of other researchers (Conrad et al. 2011). More studies with a high number of isolates are necessary to evaluate if any correlation exists between MS length and biological or clinical features.

Conclusions

In the studied population, the prevalence of *T. vaginalis* was low. Nonetheless, a remarkable increased tendency was detected in the last 2 years in general population and especially in pregnant women. To our knowledge, our study is the first in Spain that evaluates the prevalence of *T. vaginalis* in general population, and at the same time includes the percentage of pregnant women over the total cases of trichomoniasis. Besides, we analyze other risk factors like the presence of urogenital pathogens in this population. Our data support previous evidence of relationship between *T. vaginalis* and mycoplasmas, both recently considered as possible endobionts of the parasite. Notwithstanding, our study has several limitations that need to be considered when interpreting the data. Further studies, including a higher number of isolates, are highly recommended in order to analyze the effect of endobionts in the clinical presentation of trichomoniasis or drug resistance. The same occurs with the microsatellite's instability observed in this study. Despite these limitations, these results support the need of including *T. vaginalis* detection in routine gynecological revisions with special emphasis in childbearing age women. Furthermore, a rescreening should be mandatory in pregnant population in order to avoid evitable risks related to *T. vaginalis* infection during pregnancy and labor as has been demonstrated in the present study.

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Compliance with ethical standards

The study protocol was carried out according to the Declaration of Helsinki. The hospital's ethics committee "Comité Ético de Investigación con Medicamentos del Hospital Universitario Puerta de Hierro Majadahonda-Madrid (CEIm)" granted permission (Acta n° 21.17) to access to medical records and use them for this study.

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

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