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Prevalence and genetic diversity of *Trichomonas vaginalis* in the general population of Granada and co-infections with *Gardnerella vaginalis* and *Candida* species

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Abstract

Purpose. Purulent or exudative genitourinary infections are a frequent cause of consultation in primary and specialized healthcare. The objectives of this study were: to determine the prevalence of *Trichomonas vaginalis* and co-infections with *Candida spp.* and *Gardnerella vaginalis* in vaginal secretion; and to use multilocus sequence typing (MLST) to analyse the genetic diversity of *T. vaginalis* strains.

Methodology. The samples were submitted for analysis (*n*=5230) to a third-level hospital in Granada (Southern Spain) between 2011 and 2014; eight *T. vaginalis* strains isolated during 2015 were randomly selected for MLST analysis. Culture and nucleic acid hybridization techniques were used to detect microorganisms in the samples.

Results. The prevalence of *T. vaginalis* was 2.4 % between 2011 and 2014, being higher during the first few months of both 2011 and 2012. Among samples positive for *T. vaginalis*, co-infection with *G. vaginalis* was detected in 29 samples and co-infection with *Candida* spp. in 6, while co-infection with all three pathogens was observed in 3 samples. The only statistically significant between-year difference in co-infection rates was observed for *T. vaginalis* with *G. vaginalis* due to an elevated rate in 2011. MLST analysis results demonstrated a high genetic variability among strains circulating in our setting.

Conclusion. These findings emphasize the need for the routine application of diagnostic procedures to avoid the spread of this sexually transmitted infection.

INTRODUCTION

Trichomonas vaginalis infection was first described as a venereal disease in the mid-20th century and is currently the most prevalent non-viral sexually transmitted infection (STI) [1]. The incidence of *T. vaginalis* is estimated by the WHO to range between 170 and 190 million cases per year worldwide [2], with vaginitis accounting for 15–20 % of these cases depending on the geographical area. Sexual intercourse is considered the main infection mechanism, explaining its designation as an STI [3]. The prevalence of *T. vaginalis* has been studied in various populations [4–6] but is known to be underestimated due to the high frequency of asymptomatic infections [2]. In the USA, the estimated prevalence fell from 22 % in 1990 to 3.9 % in 2004 [7], while other authors described a prevalence of 3.1 % among women of reproductive age between 2001 and 2004 [8]. This highly prevalent infection has not traditionally been included in screening programs on the grounds that it is a self-limiting disorder. However, it can persist for at least three months if untreated [9] and has been associated with reproductive tract complications, including pelvic inflammatory disease and adverse pregnancy outcomes [10–13]. Vaginal infection by *T. vaginalis,* among other treatable infections, can disrupt the balance of the vaginal ecosystem and impair innate protective mechanisms against infection, increasing the risk of more serious human

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Abbreviations: HIV, human immunodeficiency virus; MLST, multilocus sequence typing; ST, sequence type; STI, sexually transmitted infection.

Sequences were assembled and edited using Unipro UGene multiplatform software 1.25 (Novosibirsk, Russia) (GenBank accession numbers: KI596667 to KI596674 and KI614004 to KI614051). The concatenated sequences were compared with data from other MLST-characterised isolates available from https://pubmlst.org/tvaginalis/ to analyse the population structure, and allele names were assigned to each sequence. Sequence types (STs) were assigned based on allelic profiles described by Cornelius *et al.* in the MLST database.

papillomavirus infections [14]. *T. vaginalis* has also been associated with an increased risk of chronic prostatitis and prostate cancer in males [15] and of human immunodeficiency virus (HIV) among female sex workers [16] and family planning clinic attendees [17] in Africa. Given that *T. vaginalis* has also been associated with an increased HIV viral load in seminal and cervicovaginal compartments, control of this infection may reduce the transmission of HIV [18]. Resistance to metronidazole has been widely reported for some years [19, 20].

Conventional testing for *T. vaginalis* is by wet mount microscopy, which offers a sensitivity of around 35 to 60 % in symptomatic women with a high bacterial load [4]. However, many asymptomatic cases, reportedly one-third of all cases in women, went undetected before the introduction of more sensitive nucleic acid amplification tests [21].

T. vaginalis isolates vary in their virulence, pathogenicity, and drug resistance, and it is therefore important to relate phenotypic variations to genotypic changes. There is a need for greater knowledge of the genetic diversity of this species, given the widespread prevalence of trichomoniasis, but there is no 'gold standard' method for genotyping *T. vaginalis* [22]. Various techniques have been used to study its genetic diversity, including isozyme analysis [23], antigen characterization [24], karyotyping [25], random amplified polymorphic DNA analysis [26], internal transcribed spacer sequencing [27], restriction fragment length polymorphism analysis [28], and microsatellite analysis [29]. However, the recently introduced multilocus sequence typing (MLST) offers the unambiguous characterization of isolates using internal sequence fragments of seven housekeeping genes [30]. MLST is a robust method that provides high levels of discrimination between isolates because specific alleles are identified and subjective interpretation is not required, allowing high throughput and standardization among laboratories [30].

In the present study, the prevalence of *T. vaginalis* was analysed in Granada, Andalusia (Spain), using data collected over four years (2011–2014). Data were also gathered on co-infection with *Candida* spp. and *Gardnerella vaginalis*. Finally, MLST was used to analyse eight *T. vaginalis* strains collected at the beginning of 2015 in order to determine the genetic diversity of *T. vaginalis* in our setting.

METHODS

Study setting

A retrospective study was performed of consecutive samples gathered from non-pregnant women between January 2011 and December 2014 in Virgen de las Nieves hospital, Granada (Southern Spain), a regional reference centre serving a population of around 440 000. A total of 5203 samples were tested for the presence of *T. vaginalis* and for co-infection with *G. vaginalis* and/or *Candida* spp. Besides pregnancy, no other exclusion criteria were applied. Samples derived from women attending the hospital with symptoms of vaginal infections, who had a mean age of 39 years. In addition, eight strains from positive cultures randomly obtained between February and May 2015 were prospectively analysed by MLST.

Sample collection

External genitals of the women were examined with a speculum to assess the flow and appearance of the vagina and cervix. Then, vaginal exudate samples were collected in a sterile single-tubed Dacron vaginal swab (Copan Lab, Italy), which was inserted into the vagina and turned three times around its axis before being removed and placed in a sterile sampling container containing Stuart medium (BioMerieux, Marcy-l'Étoile, France) for delivery to the laboratory for microbiological culture and species identification.

Microbial identification

All samples were tested within 6–12 h of their reception in the laboratory for the presence of *Trichomonas* spp., *Candida* spp., and *Gardnerella* spp. using the BD Affirm VPIII Microbial Identification System with the BD MicroProbe Processor (Becton Dickinson, USA) according to the manufacturer's instructions. This nucleic acid hybridization system directly detects clinically significant [31] concentrations of *T. vaginalis* ($\geq 5\times10^3$ c.f.u. per sample), *G. vaginalis* ($\geq 2\times10^5$ c.f.u. per sample) and the most common *Candida* species (*C. albicans*, *C. glabrata*, *C. kefyr*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*) ($\geq 1\times10^4$ c.f.u. per sample). Selected swabs were seeded in Roiron culture medium for *Trichomonas* spp. (Difco Labs, USA) to recover *Trichomonas* strains for MLST analysis.

Statistical analysis

After descriptive statistical analyses, the statistical significance of differences was determined with the contingency table chi-square test. Stata 13.1 (StataCorp, TX, USA) was used for all data analyses, considering $P<0.05$ to be statistically significant.

DNA extraction

DNA was extracted from *T. vaginalis* trophozoites recovered from cultures. Cells were pelleted by centrifugation at 1000 ***g*** for 10 min, washed in PBS to eliminate residuals of the medium, and then resuspended in 200 µl of PBS (pH 7.4). DNA was extracted using the QIAamp DNA mini kit following the manufacturer's instructions and resuspended in 100 µl of the elution buffer supplied in the kit.

MLST

MLST was used to characterize eight clinical isolates collected during 2015. Seven housekeeping genes (between 450–500 bp amplicon size) described by Cornelius *et al.* [22] were PCR-amplified for each isolate, including tryptophanase (P1), glutaminase (P3), family T2 asparaginase-like threonine peptidase (P6), alanyl-tRNA synthetase (P8), DNA mismatch repair protein (P13), serine hydroxymethyltransferase (P14) and mannose 6-phosphate isomerase (P16). Genes were amplified using GoTaq DNA polymerase under the conditions described by Cornelius *et al.* [22].

Table 1. Distribution of cases positive for *T. vaginalis* between 2011 and 2014

A summary of the results for the four years are indicated in grey at the bottom of the table.

Year		Month												Total
		January	February	March	April	May	June	July	August	September	October	November	December	
2011	n	3	7	10	1	2	4	2	0	5	1	1	4	40
	%	3%	5.88 %	8.40 %	0.96 %	2.08 %	3.10 %	2.33 %	_	5.10 %	0.76 %	0.91 %	5.19 %	3.20 %
	n	100	119	119	104	96	127	86	80	98	132	109	77	1247
2012	n	7	4	3	2	1	3	2	2	4	4	3	1	36
	%	6.90 %	4.10 %	3.30 %	2.40 %	1.10 %	3.40 %	2.40 %	2.90 %	3.80 %	3.10 %	2.50 %	1.40 %	3.20 %
	n	102	98	90	82	93	88	83	70	105	130	122	69	1132
2013	n	2	3	4	5	2	1	1	1	1	1	0	1	22
	%	1.79 %	2.46 %	3.51 %	3.76 %	1.57 %	0.79 %	0.93 %	1.20 %	1.01 %	0.74 %	_	1.32 %	1.60 %
	n	112	122	114	133	127	126	107	83	99	135	136	76	1370
2014	n	2	3	1	2	3	3	3	1	3	2	3	3	29
	%	1.70 %	2.50 %	0.80 %	1.80 %	2.20 %	2.60 %	2.80 %	1 %	2.70 %	1.30 %	1.90 %	3 %	2 %
	n	120	119	123	113	135	114	109	98	111	156	155	101	1454
Total Negative (2011–2014)	n	420	441	428	422	443	444	377	327	400	545	515	314	5076
	%	96.80 %	96.30 %	96.00 %	97.70 %	98.20 %	97.60 %	97.90 %	98.80 %	96.90 %	98.60 %	98.70 %	97.20 %	97.60 %
Total Positive (2011–2014)	n	14	17	18	10	8	11	8	4	13	8	7	9	127
	%	3.23 %	3.71 %	4.04 %	2.35 %	1.77 %	2.42 %	2.10 %	1.21 %	3.15 %	1.45 %	1.27 %	2.80 %	2.40 %
	n	434	458	446	432	451	455	385	331	413	553	522	323	5203

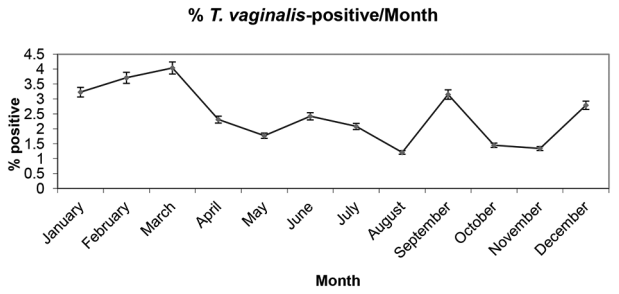

Fig. 1. Average percentage (%) of positive cases per month between 2011 and 2014. Error bars are included.

After evaluating amplicons on a 1 % agarose gel, PCR products were purified (StrataPrep PCR Purification Kit, Cat#400771, Agilent Technologies) and resuspended in 25 µl of ultrapure water. DNA was prepared for bilateral sequencing by the Sanger method at the GENYO Genomics Unit (Granada, Spain). Sequences were assembled and edited using Unipro UGene multiplatform software 1.25 (Novosibirsk, Russia) [32] (GenBank accession numbers: KI596667 to KI596674 and KI614004 to KI614051). The concatenated sequences were compared with data from other MLST-characterized isolates available from https://pubmlst.org/tvaginalis/ to analyse the population structure, and allele names were assigned to each sequence. Sequence types (STs) were assigned based on allelic profiles described by Cornelius *et al.* [22] in the MLST database.

Table 2. *Trichomonas vaginalis* (TV) prevalence and co-infection with *Gardnerella vaginalis* (GV) and/or *Candida* spp. (CA) in samples collected between 2011 and 2014. The percentage of positive samples is displayed

Year	Microorganism	Positive (n)	Total positive (n)	% positive	Total (n)
2011	TV	23	40	1.84	1247
	TV+GV	13		1.04	
	TV+CA	3		0.24	
	TV+GV+CA	1		0.08	
2012	TV	29	36	2.56	1132
	TV+GV	7		0.62	
	TV+CA	0		0	
	TV+GV+CA	0		0	
2013	TV	20	22	1.46	1370
	TV+GV	2		0.15	
	TV+CA	0		0	
	TV+GV+CA	0		0	
2014	TV	17	29	1.17	1454
	TV+GV	7		0.48	
	TV+CA	3		0.21	
	TV+GV+CA	2		0.14	
					5203

RESULTS

Epidemiological prospective study of *T. vaginalis* and co-infections with *G. vaginalis* and *Candida* spp.

A total of 5203 women were screened for *T. vaginalis*. Their mean age was 39 years (range, 17–83 years). A total of 127 positive samples were identified, a prevalence of 2.4 %. Patients were treated with 1 g metronidazole day^{-1} for 7 days. No cases were refractory to this treatment, and there were no recurrences. In 2011, 2012, 2013, and 2014, respectively, 40, 36, 22, and 29 positive samples were detected. Table 1 displays the distribution of positive samples by month. There were no statistically significant between-year differences in the number of *T. vaginalis* infections (P=0.052). Fig. 1 depicts the seasonal distribution of infections during the study period, showing a higher prevalence at the beginning of the year and during September.

The 127 *T. vaginalis*-positive samples were analysed for co-infections, finding 29 cases of co-infection with *G. vaginalis,* 6 cases of co-infection with *Candida* spp, and 3 cases of co-infection with all three pathogens (Table 2). No statistically significant differences between years were observed for *T. vaginalis*+*Candida* (P=0.86) or *T. vaginalis*+*G. vaginalis* +*Candida* (P=0.523), while a significant difference was found for *T. vaginalis*+*G. vaginalis* (P=0.023) due to a high co-infection rate (n=13) in 2011 (Table 1).

Sequence diversity

Eight strains of *T. vaginalis* isolated in the beginning of 2015 were randomly selected for analysis by MLST, amplifying and sequencing the seven loci proposed by Cornellius *et al.* [22]; these authors described 60 STs, and 47 new STs were recently published (ST-61 to ST-107) [33]. All alleles identified in the present study were already in the MLST database. Out of the eight isolates analysed, three were identified as ST-7, ST-10, and ST-32, whereas the allelic profiles of the remaining five isolates have not previously been reported [22, 33] (Table 3).

DISCUSSION

Infection with *T. vaginalis* is a frequent cause of vaginal discharge and can be responsible for pregnancy complications and an increased risk of STI [34] and human papillomavirus, among other infections, due to the production of cytotoxic enzymes and the degradation of vagina-protective components [35, 36].

In this study of a large number of samples (n=5203 from different clinical episodes) from non-pregnant women in Southern Spain, the prevalence of *T. vaginalis* infection was 2.4 %, while the simultaneous presence of infection with *Candida* spp. and/or *G. vaginalis* was detected in a substantial proportion of positive cases.

A widely varying prevalence of *T. vaginalis* has been reported in different populations from the USA [7, 8], Africa and Europe [37]. Thus, the prevalence was found to be 6 % of women studied in Europe and 18 % of those in

Table 3. MLST profiles [sequence types (STs)] identified in this study

STs numbered up to 107 were previously described [33]. New ST Numbers were assigned to the four new STs, which will be uploaded to the *T. vaginalis* database https://pubmlst.org/tvaginalis/. TRYP, tryptophenase; GLUT, glutaminase; FT2A, family T2 aparaginase-like threonine peptidase; ALTS, alanyl-tRNA synthetase; DMRP, DNA mismatch repair protein; SHMT, serine hydroxymethyltransferase; M6PI, mannose 6-phosphate isomerase.

Isolate number	MLST alleles							MLST profiles
	TRYP	GLUT	FT2A	ALTS	DMRP	SHMT	M6PI	ST
1499	1	1	1	2	11	1	2	ST-7
5523	1	1	6	6	8	1	1	ST-10
9934	1	3	6	15	5	1	2	ST-32
7826	5	3	1	4	8	1	2	ST-108
3412	1	1	1	7	5	2	2	ST-109
1659	1	3	1	4	5	1	1	ST-110
10 625	1	1	1	7	11	1	2	ST-111
10 875	5	1	6	4	5	1	2	ST-112

Africa [37], while studies between 2001 and 2004 in the USA found a prevalence of 13.3 % in African-American women but only 1.3 % in Caucasian-American women [8]. Many researchers have focused on high-risk populations (e.g. female sex workers), especially in developing countries with high STI rates [38–40]. A very high prevalence of 39.3 % (95 % CI: 26.3–52.3) was estimated for the general population of Papua New Guinea in a meta-analysis [39], with reports from the same region of a prevalence of 21.34 and 37.4 % in studies of 400 and 125 pregnant women, respectively [5, 38].

The presence of various infections, including *T. vaginalis*, *G. vaginalis* and *Chlamydia trachomatis,* has been studied in Spain, although these were not the main focus of the research [36, 41]. The prevalence of *T. vaginalis* was estimated to be 12.7 % in the general population of Spain between 1977 and 1979 [42], whereas a prevalence of only 1 % was reported in the 5–55 years age group in 2016 [43]. Sex workers in Spain showed an elevated prevalence of 17 % in 2011, and the infection was found to be strongly associated with human papillomavirus infection [36]. The reported prevalence of *T. vaginalis* in Spain decreased among males over 30 years of age from 1.4 % in 1986 to 0.9 % in 2000 and among female sex workers from 7.0 % in 1986 to 3.4 % in 2001 [44, 45]. Vaginal trichomoniasis remains one of the most frequent STIs, but there appears to have been a progressive decrease in its incidence in developed countries, with a lesser change in developing countries [45, 46]. It is acknowledged that published data underestimate the true prevalence, given the large number of individuals who are undiagnosed carriers of the infection [47].

In the present study of samples collected between 2011 and 2014, a significantly higher prevalence of *T. vaginalis* was found in the first few months of 2011 and 2012. The incubation period of this infection is unknown but was found to be from 4 to 28 days in *in vitro* studies [48]; therefore, it might be speculated that a possible increase in sexual activity during the lengthy Christmas/New Year holiday period in Spain may play a role in the higher frequency observed during the following weeks in these years.

Various authors have studied co-infections between *T. vaginalis* and other pathogens [38, 49]. In the present study, co-infection with *G. vaginalis* and/or *Candida* spp. was observed in 29.9 % of positive cases. *G. vaginalis* is a facultative anaerobe component of the human vaginal flora, that cause vaginosis when an alteration occurs in the microbial flora of the vagina [50]. However both pathogens (*Gardnerella* and *Candida*) can be acquired simultaneously by sexual contact. A significant between-year difference was only found for co-infection of *T. vaginalis* with *G. vaginalis,* due to an elevated number of these cases in 2011.

The genetic diversity of *T. vaginalis* in our setting was explored by using MLST to analyse eight positive isolates gathered at the beginning of 2015. The MLST database includes 51 different alleles and 60 STs from the study by Cornellius *et al.* [22] along with 47 subsequently reported STs [33], although 18 more recently described STs have not yet been incorporated and their profiles are not available for comparison [51]. Out of the eight strains randomly selected for study from *T. vaginalis*-positive samples gathered in 2015, three could be identified in the MLST database (ST-7, ST-10 and ST-32), whereas the allele profiles of the remaining five have not previously been described. In general, a high rate of new STs (n=126) has been reported in the 162 isolates genotyped to date [22, 33, 51], suggesting that many new STs are likely to be discovered, confirming the high genetic diversity of *T. vaginalis* [51].

Study limitations include the absence of data on the association between strain/genotype and the development of symptoms or clinical outcome, and the fact that the genetic diversity was not examined in the strains collected between 2011 and 2014.

In conclusion, a prevalence of 2.4 % was found for *T. vaginalis* in the population of Granada (Sothern Spain). Peaks in infection rates were observed in the first few months of 2011 and 2012, and co-infection with *G. vaginalis* was found

in around a quarter of positive cases. MLST analysis of samples gathered at the beginning of 2015 demonstrated a high genetic variability among strains circulating in our setting, in line with studies in other populations.

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Ethical statement
The study protocol was carried out in accordance with the Declaration of Helsinki. This was a non-interventional study based solely on routine procedures using biological material only for standard genital tract infection diagnostics as prescribed by attending physicians. There was no additional sampling or modification of the routine sampling protocol, and data analyses were carried out using an anonymous database. Therefore, ethical approval was considered unnecessary according to national guidelines. The Clinical Management Unit of Infectious Diseases and Clinical Microbiology of the University Hospital Virgen de las Nieves, Spain granted permission to access and use the data.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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