

BMJ Open Multilocus sequence typing of *Trichomonas vaginalis* clinical samples from Amsterdam, the Netherlands

C van der Veer, M Himschoot, S M Bruisten

To cite: van der Veer C, Himschoot M, Bruisten SM. Multilocus sequence typing of *Trichomonas vaginalis* clinical samples from Amsterdam, the Netherlands. *BMJ Open* 2016;**6**:e013997. doi:10.1136/bmjopen-2016-013997

► Prepublication history and additional material is available. To view please visit the journal (<http://dx.doi.org/10.1136/bmjopen-2016-013997>).

Received 24 August 2016
Accepted 1 September 2016



CrossMark

Public Health Service of Amsterdam, Public Health Laboratory, Amsterdam, The Netherlands

Correspondence to

C van der Veer;
cvdveer@ggd.amsterdam.nl

ABSTRACT

Objectives: In this cross-sectional epidemiological study we aimed to identify molecular profiles for *Trichomonas vaginalis* and to determine how these molecular profiles were related to patient demographic and clinical characteristics.

Setting: Molecular typing methods previously identified two genetically distinct subpopulations for *T. vaginalis*; however, few molecular epidemiological studies have been performed. We now increased the sensitivity of a previously described multilocus sequence typing (MLST) tool for *T. vaginalis* by using nested PCR. This enabled the typing of direct patient samples.

Participants: From January to December 2014, we collected all *T. vaginalis* positive samples as detected by routine laboratory testing. Samples from patients either came from general practitioners offices or from the sexually transmitted infections (STI) clinic in Amsterdam. Epidemiological data for the STI clinic patients were retrieved from electronic patient files.

Primary and secondary outcome measures: The primary outcome was the success rate of genotyping direct *T. vaginalis* positive samples. The secondary outcome was the relation between *T. vaginalis* genotypes and risk factors for STI.

Results: All 7 MLST loci were successfully typed for 71/87 clinical samples. The 71 typed samples came from 69 patients, the majority of whom were women (n=62; 90%) and half (n=34; 49%) were STI clinic patients. Samples segregated into a two population structure for *T. vaginalis* representing genotypes I and II. Genotype I was most common (n=40; 59.7%). STI clinic patients infected with genotype II reported more sexual partners in the preceding 6 months than patients infected with genotype I (p=0.028). No other associations for gender, age, ethnicity, urogenital discharge or co-occurring STIs with *T. vaginalis* genotype were found.

Conclusions: MLST with nested PCR is a sensitive typing method that allows typing of direct (uncultured) patient material. Genotype II is possibly more prevalent in high-risk sexual networks.

INTRODUCTION

Trichomonas vaginalis is globally the most common, non-viral, sexually transmitted

Strengths and limitations of this study

- *Trichomonas vaginalis* multilocus sequence typing (MLST) with nested PCR is a sensitive and culture independent typing method that can be used directly on patient material.
- This is the first European study in which molecular epidemiology of *T. vaginalis* was performed using both sexually transmitted infection (STI) clinic samples and samples from general practitioners within one capital city.
- With the exception of high-risk sexual behaviour, no epidemiological correlates were found with *T. vaginalis* genotype in an STI clinic population in Amsterdam, the Netherlands.
- *T. vaginalis* MLST was performed on samples from one city in the Netherlands only.
- The diversity using this MLST was so high that patients could not be linked directly but only two 'genotypes' could be discerned.

pathogen and it is estimated that some 250 million new infections occur each year.¹ *T. vaginalis*, an obligate extracellular protozoan, is the causative agent of trichomoniasis; a condition that can cause vaginitis in women and urethritis in men, but can also remain asymptomatic. Infection with *T. vaginalis* has been associated with increased risk of HIV acquisition.²

The publication of the *T. vaginalis* genome and recent advances in molecular typing methods have given scientists a better understanding of the genetic make-up of the organism, but have also provided the medical community a tool to study genetic relatedness between clinical samples.³ Such a tool potentially allows tracking of transmission routes, outbreaks and treatment failure versus reinfections. Despite these technical advances, only a few studies have looked into *T. vaginalis* genetic diversity in relation to patient demographic and clinical characteristics.⁴⁻⁷

Molecular typing studies consistently describe a two-type population structure for *T. vaginalis*, irrespective of the typing

technique that was used, as reviewed in ref. 8. *T. vaginalis* genotype I proved to be more genetically diverse and, therefore, presumably older than genotype II in evolutionary history.⁸ Surprisingly, the two genotypes seem nearly equally prevalent across the globe, except for a preponderance for type I in South Africa and type II in Mexico.⁵ Of clinical relevance, type I is more frequently infected with the pathogenic *T. vaginalis* virus (TVV) and type II has been associated with metronidazole resistance.^{5,9}

Previously, *T. vaginalis* has been typed using random amplified polymorphic DNA, restriction fragment length polymorphisms of a single gene, and 22 microsatellite markers, but these techniques are often difficult to reproduce in other laboratories.^{9–11} A more robust technique, multilocus sequence typing (MLST) was first introduced in 1998, as reviewed in ref. 12 and identifies polymorphism in approximately seven housekeeping genes through sequencing of PCR amplicons. Such an MLST was also developed for *T. vaginalis*.¹³ However, this MLST scheme requires culturing of patient material to ensure sufficient DNA load for testing.

Here we have modified the MLST method by improving sensitivity, using nested PCR, for the characterisation of *T. vaginalis* in clinical samples from the Public Health Laboratory in Amsterdam, the Netherlands. We aimed to identify molecular profiles for *T. vaginalis* and determine how these molecular profiles related to patient demographic and clinical characteristics.

MATERIALS AND METHODS

Trichomonas vaginalis clinical samples

Our study was performed at the Public Health Laboratory in Amsterdam, the Netherlands. We selected stored vaginal, urine or cervical samples from patients that had tested positive for *T. vaginalis* from January to December 2014. Samples were either collected as part of routine testing procedure at the STI outpatient clinic in Amsterdam, the Netherlands or were sent to the Public Health Laboratory by local general practitioners (GPs) for *T. vaginalis* testing. GPs and patients of the STI outpatient clinic are notified that samples sent to the Public Health Laboratory may be used for scientific research after having been anonymised. If patients object, their samples and clinical data are discarded.

T. vaginalis was detected using a transcription-mediated amplification (TMA) nucleic acid test (Hologic, Bedford, Massachusetts, USA). The remainder of the samples were stored at -20°C until further processing for this study. A data manager collected and anonymised demographic and clinical data from electronic patient files.

DNA extraction and amplification

Clinical samples used in this study were either cervical or vaginal swabs or first-catch urine that had been transferred directly into APTIMA transportation tubes

(Hologic, Bedford, Massachusetts, USA). DNA was extracted from the APTIMA transportation tubes samples by isopropanol precipitation and the pellet was dissolved in 50 μL of 10 mM Tris/HCL.¹⁴ *T. vaginalis* positives in TMA were confirmed by a real-time *T. vaginalis* PCR previously described by Schirm *et al*,¹⁵ using Phocine herpesvirus DNA as internal control. We used previously published MLST primers to amplify the seven housekeeping genes: Tryptophenase (*trp*), Glutaminase (*glut*), Family T2 aparaginase-like threonine peptidase (*ft2a*), Alanine tRNA synthetase (*alts*), DNA mismatch repair protein (*dmrp*), Serine hydroxymethyltransferase (*shmt*) and Mannose 6-phosphate isomerase (m6pi).¹³ To increase the sensitivity of the MLST method, we amplified all loci by nested PCR. We used the published primers as inner primers and developed outer primers using gene sequences from the National Center for Biotechnology Information website. The inner and outer primers are listed in table 1. We modified the published *dmrp* reverse primer, as this primer overlapped with the trimming position. Inner primers were tagged with M13 and amplicons were sent to the Amsterdam Medical Center sequencing facility for further processing. Samples with low quality sequences were reamplified and sequenced at the Public Health Laboratory using an ABI 3130 genetic analyser (Applied Biosystems) as previously described.¹⁴

MLST typing

Consensus sequences were aligned using the MLST plugin in BioNumerics V.7 (Applied Maths, Belgium, Sint-Martens-Latem) and trimmed to the specified lengths (table 1). Only samples for which all loci could be sequenced were assigned a sequence type and used in further analyses. Allele numbers and sequence types (STs) were assigned according to STs previously published.¹³ Novel alleles and STs were numbered consecutively in order of discovery, following those previously published.¹³

Population structure and clustering analyses

We entered complete MLST profiles into the Bayesian clustering program STRUCTURE 2.3.3 using the admixture model with correlated allele frequencies.¹⁶ The program calculates probabilities for the number of populations (K) that the samples belong to according to allele frequencies at each locus. For each number assumed for K (ranging from K=1 to K=10), five runs of 10^5 iterations were performed, following a burn-in period of 10^5 iterations. The value for K best describing our population was derived using the ΔK method.^{17,18} MLST profiles were also entered into a minimum spanning tree using the MLST plugin of BioNumerics V.7 (Applied Maths, Sint-Martens-Latem, Belgium), using default settings. To compare our population structure with that of others using *T. vaginalis* MLST, we included the STs of 15 reference strains in our population structure analyses.^{13,19}

Table 1 Multilocus sequence typing scheme for *Trichomonas vaginalis*

MLST locus Accession number	Primer	Sequences (5'- 3')	Gene size (bp)	5' position in gene*	Sequence length†	Total number of alleles (previously reported)‡
Tryptophanase (P1) XM_001330582.1	Outer Forward	GAGATGTTCAAGTATGCCGATG	1451	739	351	6 (6)
	Outer Reverse	CAAGCTGGACTGTGAAGTGG		1356		
	Inner Forward	CGTCAACATCGGTGGCTTCA		792		
Glutaminase (p3) XM_001295355.1	Inner Reverse	GCGACAGCGACGACATTCAT	886	1261	285	13 (5)
	Outer Forward	AAACGCTGGTGCCATTACAAC		301		
	Outer Reverse	AAGGTTCTTGCCACGGATTG		822		
Family T2 aparaginase-like threonine peptidase (P6) XM_001323020.1	Inner Forward	TCGTTAATAGTGGGTAAGGACG	990	326	318	8 (6)
	Inner Reverse	CCAAGTATAGCTCCGCTGAC		740		
	Outer Forward	AAGGCTGTTGAGCGTGGTGC		94		
Alanyl tRNA synthetase (P8) XM_001315470.1	Outer Reverse	GGTGTTCGCGCTGTAGAAGTGC	3075	560	430	20 (18)
	Inner Forward	GAACAGGAGCACCAGCAGAA		125		
	Inner Reverse	TCTCTAGCAACGCAGCCAAC		517		
DNA mismatch repair protein (P13) XM_001301638.1	Outer Forward	AAGGAGGCAGAAGACAAGTGG	1758	2167	447	14 (12)
	Outer Reverse	TGGAGCATACTTAGGAATGTTAGC		2833		
	Inner Forward	TCTGTCCAGGATGGTGTCTT		2332		
Serine hydroxymethyltransferase (P14) XM_001322558.1	Inner Reverse	ACGCCTTCCTCCTTCATCTT	1356	2806	339	2 (2)
	Outer Forward	GGACTATCCTACGATGCTATTGAG		1132		
	Outer Reverse	GTGACCAAATTTGCGACCAAG		1679		
Mannose 6-phosphate isomerase (P16) XM_001325698.1	Inner Forward	TCAAGGATATGGAAATCATCG	1128	1166	372	5 (6)
	Inner Reverse	GTCTTCCGTGCGGACAATTC		1656		
	Outer Forward	CAGGTGATACATTTATGGGATTG		383		
	Outer Reverse	GGCTATCTGTTCCCTCCACTGAC		985		
	Inner Forward	GCTGAGTGACGGTGGAACATT		408		
	Inner Reverse	GAAGATGAGGTCCTCCTTGA		837		
	Outer Forward	TTACGGTTTTTCGTCCATTTG		372		
	Outer Reverse	GACAACAACACCTCTTCCTGC		1030		
	Inner Forward	AGCCAGTTGGCTTCTGAGTT		538		
	Inner Reverse	AACAATTCCGCAAGCTGGAG		977		

Italics: previously published, Cornelius *et al.*¹³

*As the exact chromosomal location of each MLST gene is unknown, positions are given in base pairs of each MLST gene relative to the reference sequence G3 (GenBank accession no: NW_001517113.1).

†Sequences were trimmed to the previously defined length, specified per locus.¹³

‡The total number of alleles found for each locus in this study. Total numbers of alleles found previously are given in brackets.
bp, base pairs; MLST, multilocus sequence typing.

Statistical analyses

All statistical analyses were performed in SPSS Statistics software V.21 (IBM). Differences in study population characteristics were tested using the χ^2 , Fisher-exact (when the expected value was <5) or Mann-Whitney U test, as appropriate.

RESULTS

T. vaginalis clinical samples and study population

Approximately 3% (n=113/3804) of samples that were tested for *T. vaginalis* in 2014 were found positive (Public Health Laboratory yearly report 2014, <http://www.ggd.amsterdam.nl/ggd/publicaties/jaarverslagen>);

87 of these *T. vaginalis* positive samples, from 78 patients, were available for this study. Seventy-one (81.6%) of these samples, from 68 patients, were successfully typed for all 7 MLST loci and included in further analyses. Of the successfully typed samples, 34 came from 34 patients of the STI outpatient clinic and 37 samples came from 34 GP patients. One GP patient was sampled twice, 6 weeks apart and was included as two separate cases in the analyses. Two GP patients provided both a cervical and a urine sample at the time of testing; only one sample per patient was included in the analyses. Most study participants were women (61/68; 91%). Six of the 7 men included in the study were STI outpatient clinic patients. The median age of the total population was 31 years (IQR: 24.0–41.5); however, the STI clinic population was younger (median, 27 years (IQR: 23–33)) than the GP population (median, 35 years old (29–42); $p=0.004$). Demographic and clinical data were only available for the STI clinic population and these are listed in tables 2 and 3. The STI clinic population comprised mostly Surinamese ($n=20$; 61.8%); other ethnicities were: Dutch Antillean, North African and (non-Dutch) European (each $n=3$; 8.8%), Dutch ($n=2$; 5.9%) and finally Sub-Sahara African and Turkish (each $n=1$; 2.9%).

MLST profiles

We identified 6 previously described STs and 47 new STs (see online supplementary table S1). Of the 55 identified alleles, 14 had not been described before (table 1). Two patients provided a cervical and a urine sample at the time of testing and the MLST profiles were identical between samples from the same patient; this finding contributes to internal validation of the MLST profiling method. Using the current MLST method it was not possible to identify mixed infections.

T. vaginalis population structure

A two-type population structure was identified using the ΔK method on the probabilities calculated by STRUCTURE per assumed number of populations K (figure 1). Samples were assigned to a subpopulation if they shared at least 80% ancestry with that subpopulation. A two-population structure best fitted our data that resembled the type I and type II subpopulations previously described.^{10 13 19} STs of reference samples were included in our analyses to compare our population assignment with that of others.^{13 19} In this way we deduced that the larger subpopulation was type I and the smaller population was type II. Five samples (three reference and two clinical samples) shared <80% ancestry with the two populations and were not assigned (undetermined type I or II).

The minimum spanning tree (MST) also clearly displayed two major clusters and when we entered the STRUCTURE population allocations into the MST we found that the sample distribution overlapped for the two methods (figure 2). Interestingly, four of five samples that were not assigned to a population using STRUCTURE, clustered together with type I in the MST (figure 2). MLST sequence types per genotype are listed in online supplementary table S2.

T. vaginalis genotype and clinical and demographic characteristics of the patient

Two samples (one GP and one STI clinic patient's sample) shared <80% ancestry with either genotype and were excluded from the statistical analyses. Infections with *T. vaginalis* type I ($n=40$; 59.7%) occurred more frequently than type II ($n=27$; 40.3%) in our study population. Nonetheless, their distribution was equal for almost all clinical and demographic characteristics of the patients. The *T. vaginalis* genotype was not associated with age, ethnicity or clinical

Table 2 Demographic characteristics, by *Trichomonas vaginalis* genotype, of patients attending GP clinics and the STI outpatient clinic in Amsterdam, the Netherlands, from January to December 2014

Demographic characteristics	STI clinic patients (n=34)	GP clinic patients (n=35; 37 samples)*	p Value (comparison GP and STI population)	<i>T. vaginalis</i> Type I (n=40)†	<i>T. vaginalis</i> Type II (n=27)†	p Value (comparison type I and II)
Gender			0.055			0.211
Male	6 (17.6)	1 (2.9)		2 (5.0)	4 (14.8)	
Female	28 (82.4)	34 (97.1)		38 (95.0)	23 (85.2)	
Age, median (IQR)	27 (23–33)	35 (29–42)	0.004	29 (23–40)	31 (28–42)	0.629
MLST genotype			0.989			–
Type I	20 (58.8)	20 (57.1)		–	–	
Type II	13 (38.2)	14 (40.0)		–	–	
Undetermined‡	1 (2.9)	1 (2.9)		–	–	

*One GP patient was sampled twice, 6 weeks apart, and was included as two separate cases in the analyses. Two GP patients provided both a cervical and a urine sample at time of testing; the MLST profiles were identical between samples from the same patient and only one sample per patient was included in the analyses.

†Population substructures assigned by STRUCTURE. Two samples (one GP and one STI clinic patient's sample) shared <80% ancestry from the subpopulations.

GP, general practitioner; MLST, multilocus sequence typing; STI, sexually transmitted infection.

Table 3 Demographic and clinical characteristics, by *Trichomonas vaginalis* genotype, of patients attending the STI outpatient clinic in Amsterdam, the Netherlands, from January to December 2014

	STI clinic patients (n=33)*	<i>T. vaginalis</i> Type I (n=20)	<i>T. vaginalis</i> Type II (n=13)	p Value (comparison type I and II)
<i>Demographic characteristics</i>				
Gender				0.306
Male	5 (15.2)	2 (10.0)	3 (23.1)	
Female	28 (84.8)	18 (90.0)	10 (79.9)	
Age, median (IQR)	27 (23–33)	24 (22–40)	30 (21–46)	0.624
Ethnicity				0.552
Dutch	2 (5.9)	1 (5.0)	1 (7.7)	
Other European	3 (8.8)	1 (5.0)	2 (15.4)	
Turkish	1 (2.9)	1 (5.0)	0 (0.0)	
North African	3 (8.8)	1 (5.0)	2 (15.4)	
Sub-Saharan African	1 (2.9)	0 (0.0)	1 (7.7)	
Dutch Antillean	3 (8.8)	2 (10.0)	1 (7.7)	
Surinamese	20 (61.8)	14 (70.0)	6 (46.2)	
<i>Behavioural characteristics</i>				
Number of sex partners previous 6 months				0.028
1	18 (54.5)	14 (70.0)	4 (30.8)	
2	9 (27.3)	5 (25.0)	4 (30.8)	
3 or more	6 (18.2)	1 (5.0)	5 (38.5)	
Sexual preference				0.241
Heterosexual	32 (97.0)	20 (100)	12 (92.3)	
Bisexual	1 (3.0)	0 (0.0)	1 (7.7)	
<i>Clinical characteristics</i>				
Urogenital discharge	24 (70.6)	16 (80.0)	8 (61.5)	0.337
Bacterial vaginosis by Amsel criteria†	5 (14.7)	4 (20.0)	1 (7.7)	0.625
Coinfections				
<i>Chlamydia trachomatis</i>	8 (24.2)	4 (20.0)	4 (30.8)	0.681
<i>Neisseria gonorrhoea</i>	2 (6.1)	2 (10.0)	0 (0.0)	0.508

Values in bold typeface are statistically significant at $p < 0.05$.

*Population substructures assigned by STRUCTURE. One STI clinic patient's sample shared $< 80\%$ ancestry from the subpopulations assigned by STRUCTURE and was excluded from the epidemiological analyses.

†Females only.

STI, sexually transmitted infection.

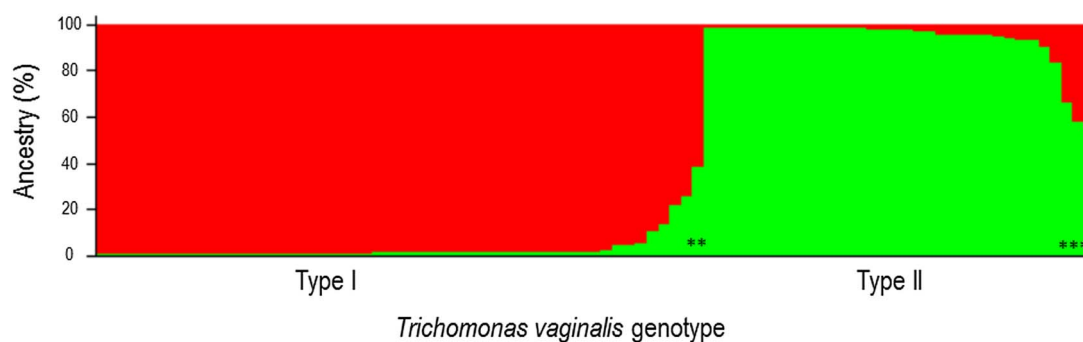


Figure 1 Assignment of clinical and reference samples by STRUCTURE. The larger subpopulation represents type I samples and the smaller subpopulation represents samples with a type II genotype. Five samples shared $< 80\%$ ancestry with the two populations and were not assigned (undetermined type I or II) and are allocated with an asterix.

characteristics of the patient, or with urogenital symptoms such as vaginal or urethral discharge. The two genotypes were equally prevalent among patients from STI and GP clinics and among males and females (see tables 2, 3 and online supplementary figures). One

significant association was noted between genotype and sexual behaviour: patients infected with type II reported more sexual partners in the preceding 6 months than patients infected with type I ($p = 0.028$; see tables 2 and 3).

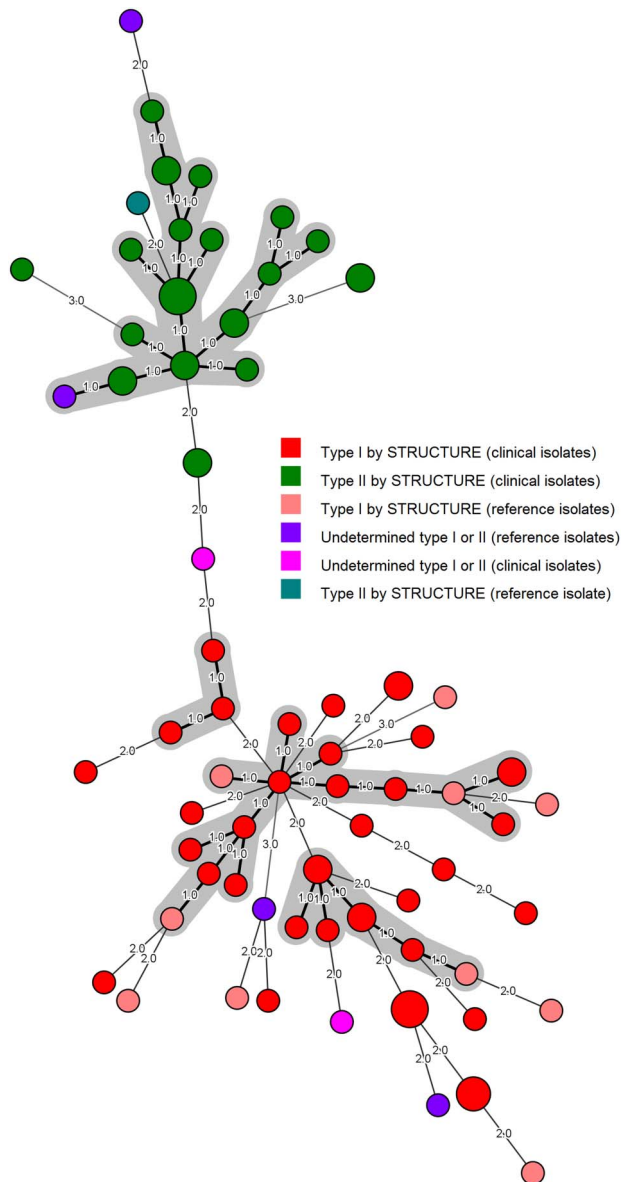


Figure 2 Clustering of clinical and reference samples in a minimum spanning tree. Clustering and dissemination of the samples in the minimum spanning tree was identical to the subpopulations assigned by STRUCTURE (figure 1), with the exception of five samples, that were not assigned to a subpopulation by STRUCTURE (<80% ancestry), clustered together with type I samples in the minimum spanning tree. Grey partitioning shows clonal complexes within the population; samples that varied by only one locus. Branch lengths correspond to the number of loci that differed between connected nodes. Larger nodes indicate samples with identical MLST profiles. MLST, multilocus sequence typing.

DISCUSSION

A previously published MLST method¹³ was successfully implemented to characterise *T. vaginalis* samples from Amsterdam, the Netherlands. By performing nested PCRs, we enhanced the MLST sensitivity and were able to use patient material that did not have to be cultured first. Similar to previous findings, two

T. vaginalis subtypes emerged in our data and inclusion of STs from reference strains confirmed the previously described type I and II subpopulations.^{7 9–11 13 19}

Apart from a positive association between type II and number of recent sex partners, no associations were seen between *T. vaginalis* genotype and patient characteristics.

T. vaginalis prevalence varies greatly per region and per population, with developing countries and minorities in developed countries being most at risk. Around 6% of women in Europe, compared to 18% of women in Africa, are estimated to harbour a *T. vaginalis* infection.²⁰ African-American women are disproportionately affected by *T. vaginalis* with a prevalence of 13.3%, compared to only 1.3% of Caucasian Americans.²¹ Also, unlike other STIs, *T. vaginalis* prevalence is positively associated with age.^{1 22} Our study was not set up as a prevalence study, yet the patient demographics in our study likely reflect the distribution of *T. vaginalis* cases in Amsterdam. In our data, we also see that most cases were Surinamese—a minority population in the Netherlands originating from Suriname, a former Dutch colony. Most cases were women and most cases were older than 30 years. Prevalence data on men are scarce since they are not tested very often and prevalence in men is estimated to be eightfold lower than in women.²⁰ The natural infection duration in men is unknown but is assumed to be no more than a few weeks, compared to months in women.²³ Men are also not routinely tested for *T. vaginalis* at the STI clinic in Amsterdam and the six positive STI clinic male samples were detected in a male *T. vaginalis* and *Mycoplasma genitalium* screening study performed previously in our laboratory.²⁴

Very few studies have described *T. vaginalis* genotype in relation to patient characteristics. Based on our current data it is unlikely that *T. vaginalis* genotype explains the ethnic or gender disparities that are commonly seen for *T. vaginalis* infection. *T. vaginalis* type I and II were equally distributed among patients of different ethnic origins, men and women and no association with *T. vaginalis* genotype and age was found. Conrad *et al* also found no association between *T. vaginalis* genotype and age.⁵ No previous studies have reported data on sexual risk behaviour in relation to *T. vaginalis* genotype and we now found that type II is positively associated with the number of reported sex partners in the preceding 6 months, possibly reflecting that this genotype circulates in high-risk sexual networks. Conrad *et al* assessed vaginal pH and positivity by Whiff test and found no association with *T. vaginalis* genotype.⁵ Brotman *et al*⁴ characterised the vaginal microbiota of *T. vaginalis* infected women and found *T. vaginalis* type I and II to be equally prevalent among women with different vaginal microbiota compositions, but this analysis included only seven women.

To date, the most striking association reported for *T. vaginalis* genotype is that type I seems to be more susceptible to metronidazole and is more frequently

infected with TVV than type II.^{5 25} Conrad *et al*⁵ have shown that 73% of type I *T. vaginalis* strains were infected with TVV, compared with only 2.5% of type II strains. Four different genotypes for TVV have been described globally.²⁶ TVV presence has been associated with symptoms and interestingly TVV genotype with severity of symptoms.^{26–28} In Cuba, TVV-1 was found only in mild to moderately symptomatic patients, whereas TVV-2 was only found in moderate to severely symptomatic patients.²⁷ Previous studies that have studied the two-type *T. vaginalis* population structure in relation to metronidazole susceptibility have done so using the metronidazole minimum lethal concentration (MLC) method and have found a mean MLC for type I of 76.6 µg/mL and a mean MLC of 228.4 µg/mL for type II.^{5 25} Paulish-Miller *et al*²⁵ have identified genetic markers in strains more resistant to metronidazole (SNPs leading to stop codons in genes encoding putative nitroreductases), but the authors emphasise that several mechanisms may be involved in metronidazole resistance. Nonetheless, information on metronidazole resistance is clinically relevant and has the potential to improve care in patients with treatment failure.^{5 29} Here we report only one GP patient that was sampled twice, 6 weeks apart, and we assume that the patient received metronidazole treatment between sampling. ST83 (*T. vaginalis* type II) was found in both samples, but, as we have no information on sexual behaviour; we do not know whether this was a persistent infection due to treatment failure or a reinfection.

A limitation of our study is that our sample size of patients with available clinical and demographic data was small and our population came from one (non-endemic) metropolitan region. We therefore cannot rule out that associations between *T. vaginalis* and patient characteristics may exist in other (endemic) regions. In this way, a bias for type I parasites (which are often infected with TVV) was seen in samples from South Africa, and moreover, a bias for type I parasites in HIV-positive women has also been reported.⁵ Those findings propose a possible parasite-virus-host interaction that needs to be investigated further, particularly in HIV-positive patients. No known HIV infections were present in our study population. As we worked with processed samples that could no longer be cultured, we did not set out to detect TVV and therefore do not know the prevalence or genetic make-up of TVV in our study population.

To conclude, the MLST profiling method for *T. vaginalis* is a robust typing method that can be reproduced between laboratories. By performing the MLST with nested PCRs we could use direct patient material that did not need to be cultured first. This method may allow easier collection and typing of specimens from high endemic, but low resource, regions. The molecular epidemiology of *T. vaginalis* is still in its infancy and further research is needed to fully understand transmission routes and pathogenicity of the parasite and its susceptibility to first choice drugs.

Acknowledgements The authors wish to thank Martijn van Rooijen for providing anonymised patient data and Titia Heijman for coordinating specimen collection from the STI outpatient clinic. The authors also thank Maarten Schim van der Loeff for critically reviewing the manuscript.

Contributors CvdV and SMB conceptualised the study. MH and CvdV performed the laboratory work. CvdV drafted the manuscript. All authors reviewed and approved the final manuscript.

Funding This work was financially supported by the Public Health Service of Amsterdam (GGD Amsterdam), the Netherlands.

Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All MLST data are available in the online supplementary files. For additional data contact Sylvia Bruisten: sbruisten@ggd.amsterdam.nl

Open Access This is an Open Access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

REFERENCES

- Poole DN, McClelland RS. Global epidemiology of *Trichomonas vaginalis*. *Sex Transm Infect* 2013;89:418–22.
- McClelland RS, Sangare L, Hassan WM, *et al*. Infection with *Trichomonas vaginalis* increases the risk of HIV-1 acquisition. *J Infect Dis* 2007;195:698–702.
- Carlton JM, Hirt RP, Silva JC, *et al*. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. *Science* 2007;315:207–12.
- Brotman RM, Bradford LL, Conrad M, *et al*. Association between *Trichomonas vaginalis* and vaginal bacterial community composition among reproductive-age women. *Sex Transm Dis* 2012;39:807–12.
- Conrad MD, Gorman AW, Schillinger JA, *et al*. Extensive genetic diversity, unique population structure and evidence of genetic exchange in the sexually transmitted parasite *Trichomonas vaginalis*. *PLoS Negl Trop Dis* 2012;6:e1573.
- Conrad MD, Kissinger P, Schmidt N, *et al*. Genetic diversity of *Trichomonas vaginalis* reinfection in HIV-positive women. *Sex Transm Infect* 2013;89:473–8.
- Rivera WL, Ong VA, Masalunga MC. Molecular characterization of *Trichomonas vaginalis* isolates from the Philippines. *Parasitol Res* 2009;106:105–10.
- Meade JC and Carlton JM. Genetic diversity in *Trichomonas vaginalis*. *Sex Transm Infect* 2013;89:444–8.
- Snipes LJ, Gamard PM, Narcisi EM, *et al*. Molecular epidemiology of metronidazole resistance in a population of *Trichomonas vaginalis* clinical isolates. *J Clin Microbiol* 2000;38:3004–9.
- Conrad M, Zubacova Z, Dunn LA, *et al*. Microsatellite polymorphism in the sexually transmitted human pathogen *Trichomonas vaginalis* indicates a genetically diverse parasite. *Mol Biochem Parasitol* 2011;175:30–8.
- Meade JC, de Mestral J, Stiles JK, *et al*. Genetic diversity of *Trichomonas vaginalis* clinical isolates determined by EcoRI restriction fragment length polymorphism of heat-shock protein 70 genes. *Am J Trop Med Hyg* 2009;80:245–51.
- Maiden MC, Jansen van Rensburg MJ, Bray JE, *et al*. MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat Rev Microbiol* 2013;11:728–36.
- Cornelius DC, Robinson DA, Muzny CA, *et al*. Genetic characterization of *Trichomonas vaginalis* isolates by use of multilocus sequence typing. *J Clin Microbiol* 2012;50:3293–300.
- Bom RJ, Christerson L, Schim van der Loeff MF, *et al*. Evaluation of high-resolution typing methods for *Chlamydia trachomatis* in samples from heterosexual couples. *J Clin Microbiol* 2011;49:2844–53.
- Schirm J, Bos PA, Roozeboom-Roelfsema IK, *et al*. *Trichomonas vaginalis* detection using real-time TaqMan PCR. *J Microbiol Methods* 2007;68:243–7.
- Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. *Genetics* 2000;155:945–59.



17. Earl DA, vonHoldt BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output implementing the Evanno method. *Conservation Genetics Resources* 2012;4:359–61.
18. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol* 2005;14:2611–20.
19. Hawksworth J, Levy M, Smale C, *et al*. Population structure and genetic diversity of the parasite *Trichomonas vaginalis* in Bristol, UK. *Infect Genet Evol* 2015;34:36–43.
20. World Health Organization (WHO). *Prevalence and incidence of selected curable sexually transmitted infections*. Geneva, 2005:1–38.
21. Sutton M, Sternberg M, Koumans EH, *et al*. The prevalence of *Trichomonas vaginalis* infection among reproductive-age women in the United States, 2001–2004. *Clin Infect Dis* 2007;45:1319–26.
22. Stemmer SM, Adelson ME, Trama JP, *et al*. Detection rates of *Trichomonas vaginalis*, in different age groups, using real-time polymerase chain reaction. *J Low Genit Tract Dis* 2012;16:352–7.
23. Van Der Pol B, Williams JA, Orr DP, *et al*. Prevalence, incidence, natural history, and response to treatment of *Trichomonas vaginalis* infection among adolescent women. *J Infect Dis* 2005;192: 2039–44.
24. van der Veer C, van Rooijen MS, Himschoot M, *et al*. *Trichomonas vaginalis* and *Mycoplasma genitalium*: age-specific prevalence and disease burden in men attending a sexually transmitted infections clinic in Amsterdam, the Netherlands. *Sex Transm Infect* 2016;92:83–5.
25. Paulish-Miller TE, Augustini P, Schuyler JA, *et al*. *Trichomonas vaginalis* metronidazole resistance is associated with single nucleotide polymorphisms in the nitroreductase genes ntr4Tv and ntr6Tv. *Antimicrob Agents Chemother* 2014;58:2938–43.
26. Goodman RP, Freret TS, Kula T, *et al*. Clinical isolates of *Trichomonas vaginalis* concurrently infected by strains of up to four *Trichomonasvirus* species (Family Totiviridae). *J Virol* 2011;85:4258–70.
27. Fraga J, Rojas L, Sariego I, *et al*. Species typing of Cuban *Trichomonas vaginalis* virus by RT-PCR, and association of TVV-2 with high parasite adhesion levels and high pathogenicity in patients. *Arch Virol* 2012;157:1789–95.
28. Rivera WL, Justo CA, Relucio-San Diego MA, *et al*. Detection and molecular characterization of double-stranded RNA viruses in Philippine *Trichomonas vaginalis* isolates. *J Microbiol Immunol Infect* 2015.
29. Bosserman EA, Helms DJ, Mosure DJ, *et al*. Utility of antimicrobial susceptibility testing in *Trichomonas vaginalis*-infected women with clinical treatment failure. *Sex Transm Dis* 2011;38:983–7.