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Original Article

Investigating links between *Trichomonas vaginalis*, *T. vaginalis* virus, *Mycoplasma hominis*, and metronidazole resistance

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Abstract

Introduction: *Trichomonas vaginalis* (TV) is the etiological agent of the common non-viral sexually transmitted infection (STI), trichomoniasis. TV can inherently harbour *Mycoplasma hominis* and *Trichomonas vaginalis* virus (TVV) species. Endosymbiosis of TV with *M. hominis* and *TVV* may contribute to metronidazole resistance in this pathogen. This study determined the prevalence of TVVs across clinical isolates of TV, as well as the symbiosis between TV, TVV, and *M. hominis* in relation to metronidazole resistance.

Methodology: Twenty-one clinical isolates of TV were analysed in this study. The isolates were subjected to drug susceptibility assays using varying concentrations of metronidazole. Nucleic acids (RNA and DNA) were extracted from the isolates for molecular assays. The presence of intracellular *M. hominis* was determined by 16S rRNA polymerase chain reaction (PCR) with specific primers. The presence of the individual TVVs was determined by PCR using gene specific primers with template cDNA.

Results: The prevalence of TVV and *M. hominis* were 76% (16/21) and 86% (18/21), respectively. No significant associations were observed between the presence of TVV and clinical symptoms. A significant association was noted between the coinfection of TVV4 and *M. hominis* (p = 0.014). The presence of any TVV was significantly associated with metronidazole susceptibility patterns (p = 0.012). No significant associations were noted between the coinfection of endosymbionts and metronidazole resistance.

Conclusions: The information obtained displays the ability of TV to form an endosymbiotic relationship with several microorganisms, simultaneously. Based on these findings, both endosymbionts pose no significant influence on metronidazole resistance.

Key words: *Trichomonas vaginalis*; *Trichomonas vaginalis* virus; *Mycoplasma hominis*; metronidazole.

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Introduction

T. vaginalis (TV) is a parasitic protozoan and the most common non-viral sexually transmitted infection (STI) responsible for trichomoniasis [1]. In 2021, the World Health Organization (WHO) estimated that there were approximately 156 million cases of trichomoniasis infections [2]. The only known host for TV is humans and the infection is spread by sexual intercourse [3]. In women, the infection persists for long periods of time ranging from months to even years, whereas in men they persist for less than 10 days [4].

Although TV infection is asymptomatic in the majority of women, trichomoniasis can present with genital signs and symptoms, such as abundant vaginal secretions with a foul odour, inflammation of the vagina accompanied by burning, itching and yellow to greenish secretions, redness and inflammation around the labia and its surrounding areas [3]. Common symptoms include frequent urination with pain, dysuria, urethritis, cystitis, cervicitis, itching and burning of the vulvar region, dyspareunia, swollen labia, and vaginal secretions [4]. Older age in women has also been associated with TV infections.

TV can be detected through either a microscopic examination of a wet mount of vaginal fluid or by highly sensitive nucleic acid amplification tests (NAAT) [7–10]. The current treatment recommended by the Centers for Disease Control and Prevention (CDC) and WHO for TV infections are metronidazole (MTZ) and tinidazole [11,12].

A double-stranded RNA (dsRNA) virus, first identified in 1985, has been observed to infect TV [13]. This virus, termed *Trichomonas vaginalis* virus (TVV), belongs to the viral family Totiviridae, is non-segmented, 4.5–5.0 kilo-base pair (kbp) in size, and is enclosed within an 85 kilodalton major viral protein capsid in a 120-subunit icosahedral configuration [14]. The TVV genetic material is replicated using viral RNA-dependant RNA polymerase. The overall protein expression of TV is affected by the presence of TVV [15,16]. Specific examples of this are the expression of the immunogenic protein P270 and cysteine

proteinases, which are associated with host immune evasion, cytotoxicity, cytoadherence, and degradation of basement membrane components produced by the vaginal epithelial cells [17]. There are 4 different viral isolates of TVV (TVV1, TVV2, TVV3 and TVV4) which can co-infect TV simultaneously, with each of these having different effects on numerous aspects of TV pathogenesis [18,19].

Carriers of either one of the TVVs have been suggested to upregulate pro-inflammatory host responses [20]. In terms of clinical symptoms experienced with TVV, correlations between specific symptoms such as dysuria, erythema, and vaginal discharge have been identified, when TVV is present in TV [21]. Specifically, TVV1 and TVV2 have been associated with genital symptom severity, whilst TVV2 and TVV3 have been correlated with virulence factors of TV and surface expression of P270 [22,23].

Recently, Austin *et al*. performed viral sequence mining in publicly available transcriptomes across 60 RNA-seq accessions that represented a minimum of 13 distinct *TV* isolates [24]. Thereafter, using a strategy known as de novo sequence assembly followed by taxonomic classification, they discovered six isolates of the newly identified fifth species known as *Trichomonas vaginalis* virus 5 (TVV5) [24]. These newly discovered isolates show high sequence identity to each other but low sequence identity to isolates of the other four species [24].

Additionally, some previous reports show that trichomonads harbouring TVV have shown an increased susceptibility to metronidazole [25]. In addition to TVV infection, some TV isolates can be naturally infected with *Mycoplasma hominis*, an intracellular bacterium that usually inhabits the lower genital tract [26]. Previous studies have speculated the possible role for coinfection of *M. hominis* and TVVs in the increase of metronidazole resistance [27]. Additionally, strong associations between *M. hominis* coinfection and metronidazole resistance in vitro have been proposed [28].

It is uncertain to what extent TVV interferes with clinical response to therapy and whether it modifies trichomonal virulence. The prevalence of endosymbionts, TVV and *M. hominis*, and their association in TV pathogenesis in a South African cohort is not yet known. Therefore, the aim of this study was to determine the prevalence of TVVs across clinical isolates of TV, as well as determine the symbiosis between TV, TVVs, and *M. hominis* in relation to metronidazole resistance.

Methodology

Culturing of TV isolates from storage

A total of 21 stored TV isolates were available for testing in this study. These isolates were obtained from a cross sectional study which included 362 pregnant women who were recruited during October 2018–March 2019 from the King Edward VIII hospital in Durban. The women participating in this study were ≥ 18 years old. They were willing to provide written consent and willing to provide a self-collected vaginal swab to be tested for TV infection. The laboratory testing and storage of the TV isolates were performed at The School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal. The isolates were stored at -70 °C until further use. For the current study, the isolates were thawed and grown in tubes containing Diamonds TYM media (BiORAD, Hercules, USA) composed of 20 g Baltimore Biological Laboratory (BBL) trypticase peptone, 10 g yeast extract, 5 g maltose, 1 g/L cysteine hydrochloride, 0.2 g L-ascorbic acid, 0.5 g agar, 900 mL distilled water, and 100 mL of heat inactivated donor horse serum). Diamonds TYM medium was supplemented with amikacin (4 µg/mL), amphotericin (5 µg/mL), ampicillin (1 mg/mL), chloramphenicol (1 µg/mL), ciprofloxacin (2 µg/mL), and vancomycin (5 µg/mL) to prevent the growth of unwanted microbes. The inoculated media were incubated at 37 °C and examined by wet mount microscopy after 24–48 hours until axenic cultures were obtained. The axenic cultures were then subjected to drug susceptibility testing, RNA extraction, cDNA synthesis, and genomic DNA extraction.

Drug susceptibility testing

Metronidazole susceptibility testing was performed in 96 well flat-bottomed microtiter plates under anaerobic incubation conditions. Two-fold serial dilutions of metronidazole was performed in Diamond's TYM medium (BiORAD, Hercules, USA). A standardized inoculum of 1.5×10^4 of trichomonads/well was used in the assay. Each TV isolate was added into a distinct well, excluding the ATCC control wells. The TV ATCC 50148 isolate was used as a control isolate and untreated cultures of the respective isolate were used as growth controls. The plates were incubated in air-tight anaerobic jars containing Oxoid™ AnaeroGen™ 2.5 L gas pack (BiORAD, Hercules, USA) and Oxoid™ Resazurin Anaerobic indicator strips (BiORAD, Hercules, USA) at 37 °C for 48 hours. TV motility and growth was assessed using the inverted microscope at × 400

magnification. TV growth and motility was scored according to the scoring criteria described by Upcroft and Upcroft [29]. Trophozoite numbers were scored as 1+ (0–10 motile parasites; not more than 20% coverage of well surface, and significantly less active); 2+ (20–50% coverage of the well surface and some trophozoite motility); 3+ (> 50 % coverage of the well surface, almost confluent growth with much motility); and 4+ (confluent growth with full motility). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of metronidazole in which a score of 1+ was observed after 48 hours of incubation. Breakpoints suggested by Upcroft and Upcroft [29] were used. MIC ≤ 1 µg/mL was considered susceptible; MIC = 2 µg/mL was considered intermediate (low-level resistance); and MIC ≥ 4 µg/mL was considered resistant. All experiments were performed in triplicate for each TV isolate.

RNA extraction

Total RNA was extracted from the cell pellets using the PureLink RNA Mini Kit (BiORAD, Hercules, USA), according to the manufacturer's instructions. Cells were transferred to a 15 mL tube and centrifuged at 2,000 × g for 5 minutes at 4 ℃ to pellet the cells. The kit instructions were then followed without any modifications. The purified RNA was then stored on ice and converted immediately to cDNA.

Synthesis of complementary DNA

The extracted RNA (7 µL) was converted to cDNA using the Maxima H Minus cDNA synthesis Master Mix with dsDNase ((BiORAD, Hercules, USA) according to the manufacturer's instructions. A nanodrop was used to measure the A260/A280 ratio and concentration (ng/µL) of the cDNA. The converted cDNA was stored at -20 °C until further use.

PCR amplification of the TVV

The four TVVs were amplified using the primers listed in Table 1. The amplification reactions were performed using the DreamTaq (BiORAD, Hercules, USA) 2X PCR master mix according to the manufacturer's instructions. A 10 µL reaction mixture was made using the 0.3 µL of the forward and reverse primers, 3.4 µL of water, 5 µL of PowerUp (BiORAD, Hercules, USA), and 1 µL of cDNA. The amplification products were resolved on a 1% agarose gel and viewed under a UV transilluminator.

DNA extraction from cultured isolates

To eliminate contamination by *Mycoplasma*, TV isolates were cultured in a medium called Diamond's TYM medium (BiORAD, Hercules, USA) which was supplemented with 50 µg/mL of gentamicin. Thereafter, sub-cultures were done daily for seven days or until axenic cultures were obtained. Extractions of DNA from TV axenic cultures were performed using the phenol-chloroform method [30]. The cultures were centrifuged twice for 10 min; thereafter cells were washed twice with phosphate buffer saline (PBS); followed by centrifugation at 1500 x g for 10 min. Lysis buffer (500 µL) was added to the TV cells and the solution was transferred to Eppendorf tubes. The tubes were then incubated at 65 °C for 30 min and left to cool at room temperature. DNA was then purified by adding equal volumes of phenol chloroform (1:1). The solution was centrifuged at 1500 × g for 10 min and the aqueous phase was transferred into a clean tube. The above-mentioned purification step was done twice. DNA was then precipitated by adding 2× volumes of 95% ethanol and 0.1 volume of 3M sodium acetate, followed by centrifugation at 1500 × g for 10 min. The supernatant was discarded, and the pellet was left to dry for ± 2 hours at room temperature. Finally, the DNA was dissolved in 50 µL TE buffer. The DNA purity and concentration was then measured using a nanodrop.

Detection of M. hominis within TV isolates

The presence of the 16S rRNA gene from *M. hominis* was investigated using TV DNA as a template. The following *M. hominis* specific primers, were used to identify TV isolates infected with *M. hominis*: forward 5'-ATACATCGATGTCGAGCGAG-3' and reverse 5'-CATCTTTTAGTGGCGCCTTAC-3'. PCR was performed in a total volume of 50 µL. The reaction

Table 1. Primers used for TVVs detection.

Primer Name	Sequence 5'-3'
TVV1 forward	ATTAGCGGTGTTTGTGATGCA
TVV1 reverse	CTATCTTGCCATCCTGACTC
TVV2 forward	GCTTGAGCACTGCTCGCG
TVV2 reverse	TCTCTTTTGGCATCGCTT
TVV3 forward	AAATTAATCAACACCCTCC
TVV3 reverse	CAGATCACTTTGTGTGTC
TVV4 forward	ATGCCAGTTGCTTTCCG
TVV4 reverse	TTCCCCAATAGTTATCAG

TVV: *Trichomonas vaginalis* virus.

mixture contained 16 µL of nuclease-free PCR water, 25 µL of the DreamTaq PCR Master Mix (BiORAD, Hercules, USA), 2 µL of each primer, and 5 µL of template DNA. The cycling conditions were: initial denaturation at 95 °C for 3 minutes; followed by 30 cycles at 95 °C for 1 minute, 53 °C for 30s, 72 °C for 1 minute; and a final extension at 72 °C for 5 minutes. PCR amplification was performed in a T100 thermocycler (BiORAD, Hercules, USA). The PCR products were analysed by electrophoresis on a 1% agarose gel in 0.5X TBE buffer at 80 V and viewed under a UV illumination system (Gene Genius System, BiORAD, Hercules, USA).

Statistical analysis

Associations between TVV and selected demographic and clinical characteristics, and metronidazole resistance were examined using Chi-square and t test statistics. All analyses were conducted using R Studio Computing [23].

Results

Metronidazole susceptibility patterns of TV isolates

The anaerobic MICs for metronidazole ranged from 0.25 to 4 µg/mL, and the mean MICs ± standard deviation was 1.63 ± 0.95 µg/mL. Of the 21 TV isolates tested, 9.5% (2/21) had an MIC of 4 µg/mL (resistant), 38.1% (8/21) had an MIC of 2 µg/mL (intermediate), and 52.4% (11/21) had an MIC ≤ 1 µg/mL (susceptible) (Table 2).

Table 2. *T. vaginalis* metronidazole susceptibility results

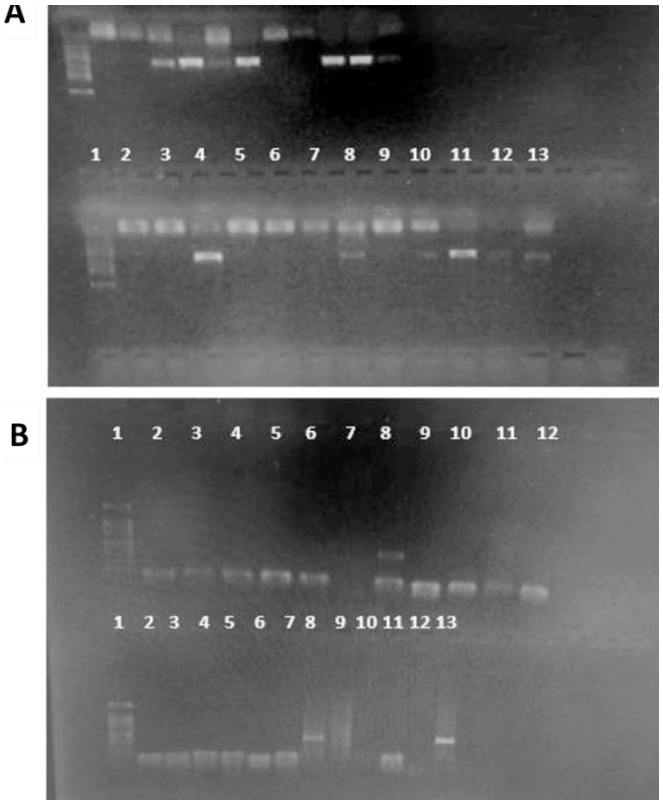
T. vaginalis isolate	Metronidazole MIC (µg/ml)	Susceptibility profile
TV101	2	Intermediate
TV128	1	Susceptible
TV171	1	Susceptible
TV179	1	Susceptible
TV182	2	Intermediate
TV184	1	Susceptible
TV209	2	Intermediate
TV211	2	Intermediate
TV230	2	Intermediate
TV231	1	Susceptible
TV233	2	Intermediate
TV241	2	Intermediate
TV253	4	Resistant
TV266	1	Susceptible
TV270	4	Resistant
TV275	1	Susceptible
TV302	1	Susceptible
TV329	1	Susceptible
TV341	2	Intermediate
TV357	1	Susceptible
TV358	0.25	Susceptible

MIC: Minimum inhibitory concentration.

Prevalence of TVVs and intracellular M. hominis

Figure 1 shows the gel images of the individual TVVs amplified across the clinical isolates of TV. The size of the PCR amplicons was 400 bp. The most prevalent TVV was TVV1, which was detected in 12 out of the 21 isolates tested (57.1%); and the second most prevalent virus was TVV2, which was detected in 11 isolates (52.3%). TVV3 and TVV4 were detected in 3 (14.2%) and 2 (9.52%) isolates, respectively. Additionally, 27 viruses were identified in 16 TV isolates, with more than one TVV detected in 10 isolates. Out of 21 *Trichomonas vaginalis* (TV) isolates screened for *M. hominis* (Figure 2), 18 harbored intracellular *M. hominis*, with a prevalence rate of 85.7%. The expected 200 bp amplicon size, corresponding to the 16S rRNA gene of *M. hominis*, was detected in these 18 isolates. Lane analysis of the

Figure 1. Agarose gels representing TV isolates screened for TVV.



A. shows TVV1 and **B.** shows TVV3. The TVV1 isolate was present in 12/21 samples (57.14%) whereas 9/21 samples (42.86%) showed no amplification for TVV1. In the case of the TVV3 isolate 3/21 samples (14.29%) showed positive amplification and 18/21 samples (85.7%) did not show any amplification. The estimated band size of the virus's presence (TVV positive) is 400bp. **A.** Lane 1-21: Individual samples screened for TVV1; 12/21 samples: Positive for TVV1 amplification (band at 400bp); 9/21 samples: Negative for TVV1 amplification (no band at 400bp). **B.** Lane 1-21: individual samples screened for TVV3; 3/21 samples: positive for TVV3 amplification (band at 400bp); 18/21 samples: negative for TVV3 amplification (no band at 400bp). TV: Trichomonas vaginalis; TVV: Trichomonas vaginalis virus.

isolates showed that 18/21 were positive for *M. hominis*, displaying a band at 200 bp, while 3/21 isolates were negative, showing no band at this marker.

Association between M. hominis infection, presence of TVVs, and metronidazole susceptibility patterns

The highest proportion of isolates that tested positive for intracellular *M. hominis* (100%), were classified as having intermediate resistance to metronidazole when compared to 50% that were classified as being resistant, and 81.8% that were classified as being susceptible. There was no significant association between the presence of intracellular *M. hominis* and metronidazole susceptibility patterns (p = 0.181). This indicates that symbiosis between *M. hominis* and TV does not have a contributory role towards metronidazole resistance in TV (Table 3).

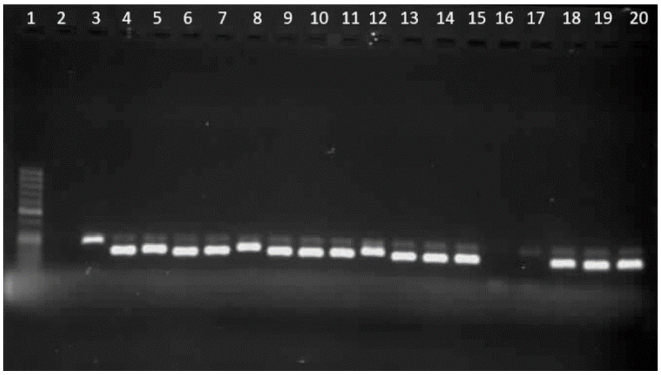
Overall, the presence of any one of the four TVVs was significantly associated with metronidazole susceptibility patterns (p = 0.012). Despite the highest frequency of TVV1 being present in metronidazole resistant isolates (72.7%) when compared to intermediate (37.5%) and susceptible isolates (50%), this association was not significant (p = 0.381). Similarly, for TVV2, TVV3, and TVV4, the highest frequencies of these viruses were shown to be present in metronidazole resistant isolates; however this was not significant ($p > 0.05$). This indicates that symbiosis between TVVs and TV does not have a significant contribution towards metronidazole resistance in TV (Table 3).

Association between M. hominis infection and the presence of TVV

Overall, there was no significant association between co-infection of *M. hominis* and TVVs (p = 0.549). However, when stratified by individual TTVs, of the isolates that tested positive for TVV4, none of them had been co-infected with *M. hominis*. This shows an inverse relationship between TVV and *M. hominis* and this was significant (p = 0.014, Table 4).

In order to determine if previous treatment for sexually transmitted infections would have provided a selective pressure for the acquisition of TVVs, the association between past treatment and the presence of

Figure 2. Agarose gel representing TV isolates screened for M. hominis.



Of the 21 isolates: 18 isolates harboured intracellular M. hominis. The expected amplicon size of 200 bp corresponding to the 16S rRNA from M. hominis was detected in the 18 isolates. The prevalence of M. hominis was 85.7%.

Lane 1-21: individual TV isolates screened for M. hominis; 18/21 isolates: positive for M. hominis (band at 200bp); 3/21 isolates: negative for M. hominis (no band at 200bp).TV: Trichomonas vaginalis; TVV: Trichomonas vaginalis virus.

Table 3. Detection of *M. hominis* and *T. vaginalis viruses* (TVV) stratified by metronidazole susceptibility patterns.

Metronidazole susceptibility patterns	Intermediate (N = 8)	Resistant (N = 2)	Susceptible (N = 11)	*p* value	Overall (N = 21)
M. hominis status				0.181	
Negative	0 (0.0%)	1 (50.0%)	2 (18.2%)	Fisher's	3 (14.3%)
Positive	8 (100.0%)	1 (50.0%)	9 (81.8%)		18 (85.7%)
TVV				0.012	
Negative	4 (50.0%)	1 (50.0%)	0 (0.0%)	Fisher's	5 (23.8%)
Positive	4 (50.0%)	1 (50.0%)	11 (100.0%)		16 (76.2%)
TVV1				0.381	
Negative	5 (62.5%)	1 (50.0%)	3 (27.3%)	Fisher's	9 (42.9%)
Positive	3 (37.5%)	1 (50.0%)	8 (72.7%)		12 (57.1%)
TVV2				0.120	
Negative	6 (75.0%)	1 (50.0%)	3 (27.3%)	Fisher's	10 (47.6%)
Positive	2 (25.0%)	1 (50.0%)	8 (72.7%)		11 (52.4%)
TVV3				1.000	
Negative	7 (87.5%)	2 (100.0%)	9 (81.8%)	Fisher's	18 (85.7%)
Positive	1 (12.5%)	0 (0.0%)	2 (18.2%)		3 (14.3%)
TVV4				0.581	
Negative	8 (100.0%)	2 (100.0%)	9 (81.8%)	Fisher's	19 (90.5%)
Positive	0 (0.0%)	0 (0.0%)	2 (18.2%)		2 (9.5%)

% and *p* values based on non-missing cases.

TVVs was determined. As shown in Table 4 and 5, neither the overall presence of any one of the four TVVs nor any of the individual TVVs were significantly associated with past symptoms or the past treatment of STIs ($p > 0.05$). This indicates that previous exposure to infection or past treatment with metronidazole does not provide a selective pressure for the acquisition of TVVs. In addition, having current symptoms of STIs such as abnormal vaginal discharge was not significantly associated with the presence of TVVs ($p > 0.05$) (Table 4). This reiterates that clinical factors are not the drivers for the acquisition of these viruses in TV.

The association between current abnormal vaginal discharge and the presence of TVV was investigated and the results yielded showed that testing positive for any TVV was not associated with current abnormal vaginal discharge.

Discussion

To the best of our knowledge, this was the first study in Durban, KwaZulu Natal to determine the prevalence of TVVs across clinical isolates of TV, and to determine the symbiosis between TV, TVVs and *M. hominis* in relation to metronidazole resistance in our current setting.

In this study, 76% of the isolates tested positive for at least one of the TVVs. The prevalence of TVVs found in this study is comparable to a prevalence of 81.9% (95% CI: 71.1–90.0) reported in a study in Cape Town, South Africa [31] and 75.0% (95% CI: 55.1–89.3) reported in a study in Baltimore, Maryland, USA [31]. More recent studies have reported lower carriage of TVV of 16% (95% CI: 7–34), in Turkey [33] and a higher prevalence of 44% (95% CI: 30–58) in Italy [34]. In this study, overall, 27 viruses were identified in 16 TV isolates, with more than 1 TVV detected in 10 of the isolates. TV can be infected by more than one species of TVV, simultaneously [34]. The ability of diverse viral species to cohabit a single host is common among Totivirus as they are predominantly non-cytopathic and can well-adjust in the host cell environment [20]. Rivera *et al.,* suggested that the

Table 4. Detection of *T. vaginalis viruses (*TVV) stratified by *M. hominis* infection status.

M. hominis	**Negative (N=3)**	**Positive (N=18)**	***p* value**	**Overall (N=21)**
TVV			Fisher's, $p = 0.549$	
Negative	0 (0.0%)	5 (27.8%)		5 (23.8%)
Positive	3 (100.0%)	13 (72.2%)		16 (76.2%)
TVV1			Fisher's, $p = 0.229$	
Negative	0 (0.0%)	9 (50.0%)		9 (42.9%)
Positive	3 (100.0%)	9 (50.0%)		12 (57.1%)
TVV2			Fisher's, $p = 1.000$	
Negative	1 (33.3%)	9 (50.0%)		10 (47.6%)
Positive	2 (66.7%)	9 (50.0%)		11 (52.4%)
TVV3			Fisher's, $p = 0.386$	
Negative	2 (66.7%)	16 (88.9%)		18 (85.7%)
Positive	1 (33.3%)	2 (11.1%)		3 (14.3%)
TVV4			Fisher's, $p = 0.014$	
Negative	1 (33.3%)	18 (100.0%)	0.029	19 (90.5%)
Positive	2 (66.7%)	0 (0.0%)	0.029	2 (9.5%)

% and *p* values based on non-missing cases.

Table 5. Association between past treatment for sexually transmitted infections (STIs) and the presence of *T. vaginalis viruses* (TVVs).

Previously treated for STI in the past	**No (N = 14)**	**Yes (N = 7)**	***p* value**	**Overall (N = 21)**
TVV			Fisher's, $p = 0.624$	
Negative	4 (28.6%)	1 (14.3%)		5 (23.8%)
Positive	10 (71.4%)	6 (85.7%)		16 (76.2%)
TVV1			Fisher's, $p = 0.642$	
Negative	7 (50.0%)	2 (28.6%)		9 (42.9%)
Positive	7 (50.0%)	5 (71.4%)		12 (57.1%)
TVV2			Fisher's, $p = 1.000$	
Negative	7 (50.0%)	3 (42.9%)		10 (47.6%)
Positive	7 (50.0%)	4 (57.1%)		11 (52.4%)
TVV3			Fisher's, $p = 0.247$	
Negative	13 (92.9%)	5 (71.4%)		18 (85.7%)
Positive	1 (7.1%)	2 (28.6%)		3 (14.3%)
TVV4			Fisher's, $p = 1.000$	
Negative	13 (92.9%)	6 (85.7%)		19 (90.5%)
Positive	1 (7.1%)	1 (14.3%)		2 (9.5%)

% and *p* values based on non-missing cases.

occurrence of numerous TVVs may be a result of more than one TV isolate infecting the same patient, as opposed to concurrent infections of different TVVs in a single TV cell [35]. The occurrence of numerous TVVs in a single TV cell may contribute to the upregulation of inflammatory reactions and development of trichomoniasis [34]. Similar to previous reports, TVV1 and TVV2 were the more dominant viruses in this study when compared to TVV3 and TVV4 [36]. Bahadory *et al.* reported in their systemic review and meta-analysis on the global status of TVV, that African regions have the highest prevalence of 66% (95% CI: 25–92%), of TVVs [37]. Accumulated evidence suggests that the endosymbiotic TVV may play a role in drug susceptibility of TV, and in the modulation and influence of the protozoan's virulence [38]. TVV is known to alter the expression profile of host cysteine proteases, which are responsible for promoting TV cyto-adhesion to the vaginal epithelium and mediating cytotoxicity to aid in parasite survival, and host immune evasion [39]. Previous studies have shown that TV isolates infected with TVV have a higher growth rate and are increasingly difficult to maintain in a culture [39]. Amongst the TV isolates in this study, it was noted that protozoa uninfected by viruses had a slower growth rate as compared to those that contained the virus. These results are in line with those by Rivera and colleagues [35]. In addition, Margarita *et al.* noticed that TV isolates infected by all 4 TVVs were difficult to cultivate in vitro [34].

The array of clinical symptoms caused by TV can differ from asymptomatic to serious symptoms. TVV has been frequently identified in symptomatic patients. However, numerous studies have found that there are no significant associations between TV isolates harbouring TVVs and clinical symptoms [1,40]. In this study, in order to determine if previous treatment for STIs would have provided a selective pressure for the acquisition of TVVs, the association between past treatment of STIs and the presence of TVVs was

Table 6. Association between past symptoms of sexually transmitted infections (STIs) and the presence of *T. vaginalis viruses* (TVVs).

Past STI symptoms	No (N=10)	Yes (N=11)	*p* value	Overall (N=21)
TVV			Fisher's, $p = 0.311$	
Negative	1 (10.0%)	4 (36.4%)		5 (23.8%)
Positive	9 (90.0%)	7 (63.6%)		16 (76.2%)
TVV1			Fisher's, $p = 0.387$	
Negative	3 (30.0%)	6 (54.5%)		9 (42.9%)
Positive	7 (70.0%)	5 (45.5%)		12 (57.1%)
TVV2			Fisher's, $p = 0.670$	
Negative	4 (40.0%)	6 (54.5%)		10 (47.6%)
Positive	6 (60.0%)	5 (45.5%)		11 (52.4%)
TVV3			Fisher's, $p = 0.586$	
Negative	8 (80.0%)	10 (90.9%)		18 (85.7%)
Positive	2 (20.0%)	1 (9.1%)		3 (14.3%)
TVV4			Fisher's, $p = 0.214$	
Negative	8 (80.0%)	11 (100.0%)		19 (90.5%)
Positive	2 (20.0%)	0 (0.0%)		2 (9.5%)

% and *p* values based on non-missing cases.

Table 7. Association between current symptoms of sexually transmitted infections (STIs) such as abnormal vaginal discharge and the presence of *T. vaginalis viruses* (TVVs).

Current abnormal vaginal discharge	No (N = 13)	Yes (N = 8)	*p* value	Overall (N = 21)
TVV			Fisher's, $p = 0.325$	
Negative	2 (15.4%)	3 (37.5%)		5 (23.8%)
Positive	11 (84.6%)	5 (62.5%)		16 (76.2%)
TVV1			Fisher's, $p = 0.673$	
Negative	5 (38.5%)	4 (50.0%)		9 (42.9%)
Positive	8 (61.5%)	4 (50.0%)		12 (57.1%)
TVV2			Fisher's, $p = 1.000$	
Negative	6 (46.2%)	4 (50.0%)		10 (47.6%)
Positive	7 (53.8%)	4 (50.0%)		11 (52.4%)
TVV3			Fisher's, $p = 0.257$	
Negative	10 (76.9%)	8 (100.0%)		18 (85.7%)
Positive	3 (23.1%)	0 (0.0%)		3 (14.3%)
TVV4			Fisher's, $p = 0.505$	
Negative	11 (84.6%)	8 (100.0%)		19 (90.5%)
Positive	2 (15.4%)	0 (0.0%)		2 (9.5%)

% and *p* values based on non-missing cases.

determined (Table 6). According to the analysis, neither the overall presence of any one of the four TVVs, nor any of the individual TVVs were significantly associated with past symptoms or the past treatment of STIs. We suggest that previous exposure to infection or past treatment with metronidazole does not provide a selective pressure for the acquisition of TVVs; however future studies with a larger number of isolates are needed to confirm this statement. In addition, this study showed that having current symptoms of STIs such as abnormal vaginal discharge is not significantly associated with the presence of TVVs (Table 7). This is consistent with the data from previous reports that showed that clinical factors are not the drivers for the acquisition of these viruses in TV. However, an association between TVV and clinical symptoms (vaginal discharge and erythematous), have been recognized by some studies [41]. These results may differ from the findings from the current study due to the heterogeneity in sample number and geographical areas. Previous studies showing associations between TVV and clinical symptoms have identified that TVV1 is linked to milder symptoms of dysuria and vaginal discharge, whilst TVV2 is specifically associated with more serious symptoms when compared to those described above [41]. Associations between the presence of TVV and metronidazole resistance in TV have been elucidated in previous reports [41]. This study noted that the presence of any one of the four TVVs was significantly associated with metronidazole susceptibility. However, the findings in this study showed the lack of relationship between the presence of TVV and metronidazole resistance, as no significant association was noted. This indicates that the symbiosis between TVVs and TV may not have a significant contribution towards metronidazole resistance in TV. However as mentioned previously, this finding can only be confirmed in future studies using a larger sample size of isolates.

In addition to TVV, this study evaluated the prevalence of intracellular *M. hominis* infection in TV clinical isolates and assessed whether associations exist between metronidazole resistance and coinfection with *M. hominis* and TVV. *M. hominis* can survive, multiply and invade the cytoplasm of TV, aiding in its defense mechanism during human infection [42]. In this study, 86% of the TV isolates tested positive for *M. hominis* and of this, 72% were co-infected with TVV. Previous reports have described varying prevalence rates of intracellular *M. hominis* in symbiosis with TV, based on different geographical regions. These range from as high as 92.5% in Mozambique, Angola and Italy, to as low as 20% in the United States of America [43]. The symbiosis of *M. hominis* in association with TV has significant clinical consequences and correlations with metronidazole resistance of TV. Majority of TV isolates infected with *M. hominis* were susceptible to metronidazole. This finding is similar to previous literature that showed no association between metronidazole resistance and *M. hominis* infection [27]. In terms of the co-infection of *M. hominis* and TVV, this study demonstrated that 72.2% of TV isolates harboring *M. hominis* were infected by at least one subclass of TVV; however this association was not significant. However, statistical correlation and significance was demonstrated between the presence of *M. hominis* and TVV4. This study further showed that none of the TV isolates were devoid of either *M. hominis* or TVV. This suggests that TV can adapt to symbiosis with different microorganisms to aid in its pathogenesis. Future studies are needed to confirm the role of other microorganisms in the pathogenesis of TV.

The influence of the aforementioned endosymbionts on TV resistance to metronidazole was assessed. Previous studies have reported on positive correlations between infection by either TVV or *M. hominis* with metronidazole resistance [44]. However, some reports showed a lack of correlation between the endosymbionts and metronidazole resistance [42]. Similarly, this study showed no significant association between the presence of intracellular *M. hominis* and metronidazole susceptibility patterns. Furthermore, 81.8% of the isolates that tested positive for *M. hominis*, were susceptible to metronidazole. No significant associations were seen in TV isolates infected with both endosymbionts in terms of metronidazole resistance. This suggests that the endosymbiosis between *M. hominis* and TVV may not contribute towards metronidazole resistance in TV. This suggestion advocates the importance of conducting further large-scale studies in order to provide associations between metronidazole resistance in TV and the presence of both TVV and *M. hominis.*

Conclusions

The prevalence of TVV in this study showed that majority of the TV isolates were infected with TVV. The study was limited by the small number of isolates screened for TVV, which made it challenging to assess any associations of TVV carriage on clinical signs and symptoms. TVV was not associated with clinical symptoms or metronidazole resistance. These results suggest that TVV and TV may have a commensal relationship. Regardless of the low number of TV

isolates, the presence of all individual and mixed (co-infections) TVV subtypes were detected in our isolates. This demonstrates that there is likely prominent genetic diversity of TVV in the TV isolated from women in Durban, South Africa. Additional studies with a higher number of isolates should be conducted to substantiate these results. The data obtained showed the high prevalence of TV isolates infected with different types of TVVs as well as *M. hominis*, displaying the ability of TV to establish an endosymbiotic relationship with different microorganisms, simultaneously. The presence of *M. hominis* in TV showed no association with TVV or metronidazole resistance. The findings in this study suggest the necessity for more cohort and cellular studies to understand the interaction between TV, TVV, and *M. hominis*; and assess how they influence pathogenicity, symptoms, and modification of host responses.

Authors' contributions

RG: conceptualization, writing—original draft preparation, review and editing; NSA: conceptualization, writing—review and editing, supervision; NM: review and editing. All authors have read and agreed to the published version of the manuscript

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Ethical standards

Ethical approval was obtained from the Biomedical Research Ethic Committee (BREC).

References

1. Centers for Disease Control and Prevention (nd) Trichomoniasis. Available: https://www.cdc.gov/trichomoniasis/about/index.html#:~:text=Trichomoniasis%20(or%20%22trich%22),cannot%20tell%20they%20have%20it. Accessed: 6 April 2022.
2. World Health Organization. (2021). Sexually transmitted infections (STIs). Available: https://www.who.int/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis). Accessed: 6 April. 2022.
3. Cudmore SL, Delgaty KL, Hayward-McClelland SF, Petrin DP, Garber GE (2004) Treatment of infections caused by metronidazole-resistant *Trichomonas vaginalis*. Clin Microbiol Rev 17: 783–793. doi: 10.1128/CMR.17.4.783-793.2004.
4. Bradic M, Warring SD, Tooley GE, Scheid P, Secor WE, Land KM, Huang PJ, Chen TW, Lee CC, Tang P, Sullivan SA, Carlton JM (2017) Genetic indicators of drug resistance in the highly repetitive genome of *Trichomonas vaginalis*. Genome Biol Evol 9: 1658–1672. doi: 10.1093/gbe/evx110.
5. Sood S, Mohanty S, Kapil A, Tolosa J, Mittal S (2007) InPouch TV culture for detection of *Trichomonas vaginalis*. Indian J Med Res 125: 567–571.
6. Patel EU, Gaydos CA, Packman ZR, Quinn TC, Tobian AAR (2018) Prevalence and correlates of *Trichomonas vaginalis* infection among men and women in the United States. Clin Infect Dis 67: 211–217. doi: 10.1093/cid/ciy079.
7. Schwebke JR, Hobbs MM, Taylor SN, Sena AC, Catania MG, Weinbaum BS, Johnson AD, Getman DK, Gaydos CA. (2011) Molecular testing for *Trichomonas vaginalis* in women: results from a prospective US clinical trial. J Clin Microbiol 49: 4106–4111. doi: 10.1128/JCM.01291-11.
8. Hobbs MM, Seña AC (2013) Modern diagnosis of *Trichomonas vaginalis* infection. Sex Transm Infect 89: 434–438. doi: 10.1136/sextrans-2013-051057.
9. Van Der Pol B, Williams JA, Taylor SN, Nye MB, Nye M, Fuller D, Schwebke JR, Barnes M, Gaydos CA EW 3rd. (2014) Detection of *Trichomonas vaginalis* DNA by use of self-obtained vaginal swabs with the BD ProbeTec Qx assay on the BD Viper system. J Clin Microbiol 52: 885–889. doi: 10.1128/JCM.02966-13.
10. Schwebke JR, Gaydos CA, Davis T, Marrazzo J, Furgerson D, Taylor SN, Smith B, Bachmann LH, Ackerman R, Spurrell T, Ferris D, Burnham CA, Reno H, Lebed J, Eisenberg D, Kerndt P, Philip S, Jordan J, Quigley N (2018) Clinical evaluation of the cepheid Xpert TV assay for detection of *Trichomonas vaginalis* with prospectively collected specimens from men and women. J Clin Microbiol 56: 1–7.
11. Kirkcaldy RD, Augostini P, Asbel LE, Bernstein KT, Kerani RP, Mettenbrink CJ, Pathela P, Schwebke JR, Secor WE, Workowski KA, Davis D, Braxton J, Weinstock HS (2012) *Trichomonas vaginalis* antimicrobial drug resistance in 6 US cities, STD Surveillance Network, 2009-2010. Emerg Infect Dis 18: 939–943. doi: 10.3201/eid1806.111590.
12. World Health Organization (nd) Essential medicines and health products information portal. Available: https://www.who.int/groups/expert-committee-on-selection-and-use-of-essential-medicines/essential-medicines-lists. Accessed: 22 April 2022.
13. Wang AL, Wang CC (1986)) The double-stranded RNA in *Trichomonas vaginalis* may originate from virus-like particles. Proc Natl Acad Sci USA 83: 7956–7960. doi: 10.1073/pnas.83.20.7956.

14. Goodman RP, Freret TS, Kula T, Geller AM, Talkington MW, Tang-Fernandez V, Suciu O, Demidenko AA, Ghabrial SA, Beach DH, Singh BN, Fichorova RN, Nibert ML (2011) Clinical isolates of *Trichomonas vaginalis* concurrently infected by isolates of up to four *Trichomonasvirus* species (Family Totiviridae). J Virol 85: 4258–4270. doi: 10.1128/JVI.00220-11.
15. Khoshnan A, Alderete JF (1994) *Trichomonas vaginalis* with a double-stranded RNA virus has upregulated levels of phenotypically variable immunogen mRNA. J Virol 68: 4035–4038. doi: 10.1128/jvi.68.6.4035-4038.1994.
16. Provenzano D, Khoshnan A, Alderete JF (1997) Involvement of dsRNA virus in the protein composition and growth kinetics of host *Trichomonas vaginalis*. Arch Virol 142: 939–952. doi: 10.1007/s007050050130.
17. He D, Pengtao G, Ju Y,Jianhua L, Zhang G, Xichen Z (2017) Differential protein expressions in virus-infected and uninfected *Trichomonas vaginalis*. Korean J Parasitol 55: 121–128. doi: 10.3347/kjp.2017.55.2.121.
18. Benchimol M, Chang TH, Alderete JF (2002) *Trichomonas vaginalis*: observation of coexistence of multiple viruses in the same isolate. FEMS Microbiol Lett 215: 197–201. doi: 10.1111/j.1574-6968.2002.tb11391.x.
19. Parent KN, Takagi Y, Cardone G, Olson NH, Ericsson M, Yang M, Lee YJ, Asara JM, Fichorova RN, Baker TS,Nibert ML (2013) Structure of a protozoan virus from the human genitourinary parasite *Trichomonas vaginalis*. mBio, 4: e00056. doi: 10.1128/mBio.00056-13.
20. Goodman RP, Ghabrial SA, Fichorova RN, Nibert ML (2011) *Trichomonasvirus*: a new genus of protozoan viruses in the family Totiviridae. Arch Virol 156: 171–179. doi: 10.1007/s00705-010-0832-8.
21. Flegr J, Cerkasov J, Kulda J, Tachezy J, Stokrová J (1987) The dsRNA of *Trichomonas vaginalis* is associated with virus-like particles and does not correlate with metronidazole resistance. Folia Microbiol (Praha) 32: 345–348. doi: 10.1007/BF02877224.
22. Bessarab IN, Nakajima R, Liu HW, Tai JH (2011) Identification and characterization of a type III *Trichomonas vaginalis* virus in the protozoan pathogen *Trichomonas vaginalis*. Arch Virol 156: 285–294. doi: 10.1007/s00705-010-0858-y.
23. Fraga J, Rojas L, Sariego I, Fernández-Calienes A, Nuñez FA (2012) 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 157: 1789–1795. doi: 10.1007/s00705-012-1353-4.
24. Manny AR, Hetzel CA, Mizani A, Nibert ML (2022) Discovery of a novel species of *Trichomonasvirus* in the human parasite *Trichomonas vaginalis* using transcriptome mining. Viruses 14: 548. doi: 10.3390/v14030548.
25. Wang A, Wang CC, Alderete JF (1987) *Trichomonas vaginalis* phenotypic variation occurs only among trichomonads infected with the double-stranded RNA virus. J Exp Med 166: 142–150. doi: 10.1084/jem.166.1.142.
26. Rappelli P, Carta F, Delogu G, Addis MF, Dessì D, Cappuccinelli P, Fiori PL (2001) *Mycoplasma hominis* and *Trichomonas vaginalis* symbiosis: multiplicity of infection and transmissibility of *M. hominis* to human cells. Arch Microbiol 175: 70–74. doi: 10.1007/s002030000240.
27. da Luz DB, dos Santos O, Frasson AP, Rigo GV, Macedo AJ, Tasca T (2015) High rates of double-stranded RNA viruses and *Mycoplasma hominis* in *Trichomonas vaginalis* clinical isolates in South Brazil. Infect Genet Evol 34: 181–187. doi: 10.1016/j.meegid.2015.07.005.
28. Xiao JCC, Xie LF, Fang SL, Gao MY, Zhu Y, Song LY, Zhong HM, Lun ZR (2006) Symbiosis of *Mycoplasma hominis* in *Trichomonas vaginalis* may link metronidazole resistance in vitro. Parasitol Res 100: 123–130. doi: 10.1007/s00436-006-0215-y.
29. Upcroft JA, Upcroft P (2001) Drug susceptibility testing of anaerobic protozoa. Antimicrob Agents Chemother 45: 1810–1814. doi: 10.1128/AAC.45.6.1810-1814.2001.
30. Ghatak S, Muthukumaran RB, Nachimuthu SK (2013) A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. J Biomol Tech 24: 224–231. doi: 10.7171/jbt.13-2404-001.
31. Weber B, Mapeka TM, Maahlo MA, Hoosen AA (2003) Double stranded RNA virus in South African *Trichomonas vaginalis* isolates. J Clin Pathol 56: 542–543. doi: 10.1136/jcp.56.7.542.
32. Wendel KA, Rompalo AM, Erbelding EJ, Chang TH, Alderete JF (2002) Double-stranded RNA viral infection of *Trichomonas vaginalis* infecting patients attending a sexually transmitted diseases clinic. J Infect Dis 186: 558–561. doi: 10.1086/341832.
33. Ertabaklar H, Malatyali E, Ozbay EPO, Yildiz I, Sinecen M, Ertug S, Bozdogan B and Güçlü O (2021) Microsatellite-based genotyping, analysis of population structure, presence of *Trichomonas vaginalis* virus (TVV) and *Mycoplasma hominis* in *T. vaginalis* isolates from southwest of Turkey. Iran J Parasitol: 16: 81. doi: 10.18502/ijpa.v16i1.5515.
34. Margarita V, Marongiu A, Diaz N, Dessì D, Fiori PL and Rappelli P (2019) Prevalence of double-stranded RNA virus in *Trichomonas vaginalis* isolated in Italy and association with the symbiont *Mycoplasma hominis*. Parasitol Res 118: 3565–3570. doi: 10.1007/s00436-019-06469-6.
35. Rivera WL, Justo CAC, Relucio-San Diego M, Loyola LM (2017) Detection and molecular characterization of double-stranded RNA viruses in Philippine *Trichomonas vaginalis* isolates. J Microbiol Immunol Infect 50: 669–676. doi: 10.1016/j.jmii.2015.07.016.
36. Jehee I, van der Veer C, Himschoot M, Hermans M, Bruisten S (2017) Direct detection of *Trichomonas vaginalis* virus in *Trichomonas vaginalis* positive clinical samples from The Netherlands. J. Virol Methods 250: 1–5. doi: 10.1016/j.jviromet.2017.09.007.
37. Bahadory S, Aminizadeh S, Taghipour A, Bokharaei-Salim F, Khanaliha K, Razizadeh MH, Soleimani A, Beikzadeh L, Khatami A (2021) A systematic review and meta-analysis on the global status of *Trichomonas vaginalis* virus in *Trichomonas vaginalis*. Microb Pathog 158: 105058. doi: 10.1016/j.micpath.2021.105058.
38. Malla N, Kaul P, Sehgal R, Gupta I (2011) The presence of dsRNA virus in *Trichomonas vaginalis* isolates from symptomatic and asymptomatic Indian women and its correlation with in vitro metronidazole sensitivity. Indian J Med Microbiol 29: 152. doi: 10.4103/0255-0857.81801.
39. Khanaliha K, Masoumi-Asl H, Bokharaei-Salim F, Tabatabaei A, Naghdalipoor M (2017) Double-stranded RNA viral infection of *Trichomonas vaginalis* (TVV1) in Iranian isolates. Microb Pathog 109: 56–60. doi: 10.1016/j.micpath.2017.04.032.
40. Masha SC, Cools P, Crucitti T, Sanders EJ, Vaneechoutte M (2017) Molecular typing of *Trichomonas vaginalis* isolates by actin gene sequence analysis and carriage of *T. vaginalis*

viruses. Parasit Vectors 10: 537. doi: 10.1186/s13071-017-2496-7.
41. Graves K, Ghosh A, Kissinger P, Muzny C (2019) *Trichomonas vaginalis* virus: a review of the literature. Int J STD AIDS 30: 496–504. doi: 10.1177/0956462418809767.
42. Dessì D, Rappelli P, Diaz N, Cappuccinelli P, Fiori PL (2006) *Mycoplasma hominis* and *Trichomonas vaginalis*: a unique case of symbiotic relationship between two obligate human parasites. Front Biosci 11: 2028–2034. doi: 10.2741/1944.
43. Rappelli P, Addis MF, Carta F, Fiori PL (1998) *Mycoplasma hominis* parasitism of *Trichomonas vaginalis*. Lancet 352: 1286. doi: 10.1016/S0140-6736(05)61372-4.
44. Snipes LJ, Gamard PM, Narcisi EM, Beard CB, Lehmann T, Secor WE (2000) Molecular epidemiology of metronidazole resistance in a population of *Trichomonas vaginalis* clinical isolates. J Clin Microbiol 38: 3004–3009. doi: 10.1128/JCM.38.8.3004-3009.2000.

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