Infection, Genetics and Evolution 34 (2015) 181–187

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

High rates of double-stranded RNA viruses and *Mycoplasma hominis* in *Trichomonas vaginalis* clinical isolates in South Brazil

Débora da Luz Becker [a,1], Odelta dos Santos [a,1], Amanda Piccoli Frasson [a], Graziela de Vargas Rigo [a], Alexandre José Macedo [a,b], Tiana Tasca [a,*]

[a] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90610-000 Porto Alegre, RS, Brazil
[b] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, 91501-970 Porto Alegre, Brazil

ARTICLE INFO

Article history:
Received 15 March 2015
Received in revised form 3 July 2015
Accepted 4 July 2015
Available online 6 July 2015

Keywords:
Trichomonas vaginalis
Symbionts
Metronidazole resistance
Mycoplasma hominis
Double-stranded RNA viruses

ABSTRACT

Trichomonas vaginalis is the etiological agent of trichomoniasis, the most common non-viral sexually transmitted disease (STD) in world, with 276.4 million new cases each year. *T. vaginalis* can be naturally infected with *Mycoplasma hominis* and *Trichomonasvirus* species. This study aimed to evaluate the prevalence of *T. vaginalis* infected with four distinct *T. vaginalis* viruses (TVVs) and *M. hominis* among isolates from patients in Porto Alegre city, South Brazil. An additional goal of this study was to investigate whether there is association between metronidazole resistance and the presence of *M. hominis* during TVV infection. The RNA expression level of the pyruvate ferredoxin oxidoreductase (*PFOR*) gene was also evaluated among metronidazole-resistant and metronidazole-sensitive *T. vaginalis* isolates. A total of 530 urine samples were evaluated, and 5.7% samples were positive for *T. vaginalis* infection. Among them, 4.51% were isolated from female patients and 1.12% were from male patients. Remarkably, the prevalence rates of *M. hominis* and TVV-positive *T. vaginalis* isolates were 56.7% and 90%, respectively. Most of the *T. vaginalis* isolates were metronidazole-sensitive (86.7%), and only four isolates (13.3%) were resistant. There is no statistically significant association between infection by *M. hominis* and infection by TVVs. Our results refute the hypothesis that the presence of the *M. hominis* and TVVs is enough to confer metronidazole resistance to *T. vaginalis* isolates. Additionally, the role of *PFOR* RNA expression levels in metronidazole resistance as the main mechanism of resistance to metronidazole could not be established. This study is the first report of the *T. vaginalis* infection by *M. hominis* and TVVs in a large collection of isolates from South Brazil.

1. Introduction

Trichomoniasis is caused by the flagellate protozoan *Trichomonas vaginalis* and is the most common non-viral sexually transmitted disease (STD), with an estimated incidence of 276.4 million new cases a year worldwide (WHO, 2012). This infection has been associated with serious consequences, including adverse pregnancy outcomes, infertility, predisposition to cervical cancer, and pelvic inflammatory disease (Grodstein et al., 1993; Viikki et al., 2000). Moreover, *T. vaginalis* is a cofactor in human immunodeficiency virus (HIV) transmission and acquisition, and trichomoniasis was recently associated with aggressive prostate cancers (Sutcliffe et al., 2006; Stark et al., 2009; Two et al., 2014).

Metronidazole and tinidazole are nitroimidazoles that are the two drugs of choice recommended by the Food and Drug Administration (FDA, USA) for the treatment of human trichomoniasis (Helms et al., 2008). The prevalence of resistant isolates seems to vary widely, achieving rates between 2.2% and 9.6% (Pérez et al., 2001; Schwebke and Barrientes, 2006). Increasing reports of failures in the treatment of trichomoniasis and the rising prevalence of metronidazole-resistant *T. vaginalis* isolates have occurred in recent years, supporting the evidence that resistance is on the rise (Klebanoff et al., 2001; Schwebke and Barrientes, 2006; Miller and Nyirjesy, 2011; Kissinger, 2015). Though the mechanism of metronidazole resistance in *T. vaginalis* from treatment failures is not well understood, one hypothesis is that the resistance of *T. vaginalis* to metronidazole occurs through several stages based on the significant reduction and eventual loss of activity of ferredoxin and pyruvate:ferredoxin oxidoreductase (PFOR), two enzymes involved in drug activation. Consequently, the release

* Corresponding author.
E-mail address: tiana.tasca@ufrgs.br (T. Tasca).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.meegid.2015.07.005
1567-1348/

and transport of electrons needed for the reduction of the nitro group are inhibited, and microbicide radicals are not generated (Rasoloson et al., 2002).

Some *T. vaginalis* clinical isolates can be naturally infected with *Mycoplasma hominis*, a small intracellular bacterium frequently found in the lower genital tract (Rappelli et al., 1998). The mechanism of metronidazole resistance in *T. vaginalis* is not elucidated, though some hypotheses speculate a potential role for coinfection of TVVs and *M. hominis* in the increase of metronidazole resistance. A strong relationship between *M. hominis* coinfection and metronidazole resistance *in vitro* was proposed (Xiao et al., 2006). In contrast, a lack of correlation in this association was described (Butler et al., 2010).

In addition to *M. hominis* infection, *T. vaginalis* usually harbor double-stranded RNA (dsRNA) viruses from the genus *Trichomonasvirus* of the Totiviridae family (Weber et al., 2003; Wendel et al., 2002). *T. vaginalis* viruses (TVVs) first identified by Wang and Wang (1985). TVVs form isometric viral particles that contain a nonsegmented 4500–5000 bp dsRNA genome, and the transmission of TVVs occurs upon cell division (Benchimol et al., 2002; Goodman et al., 2011a). *T. vaginalis* can be infected with up to four distinct TVVs (Goodman et al., 2011b). The precise contribution of the virus in enhancing or decreasing the virulence of this parasite is unknown, though some studies have contributed to elucidate the role of TVVs during infection. These studies suggest an association between the expression of immunogenic proteins on the trichomonad surface, variations in protozoal phenotypes, and upregulation of certain proteins which include known virulence factors (Gerhold et al., 2009; Khoshnan and Alderete, 1994).

Due to the paucity of clinical and molecular epidemiological information, the association between *T. vaginalis* pathogenesis and the presence of *M. hominis* and TVVs has not been definitively established. The discrepancies noted in previous studies highlight the need for epidemiological studies with a larger number of *T. vaginalis* isolates that will contribute to an understanding of the relationship between drug resistance and the genetic identity of *T. vaginalis* isolates. Thus, we evaluated the prevalence of TVVs and *M. hominis* infection in a large number of fresh *T. vaginalis* clinical isolates from South Brazil. We also attempted to evaluate whether an association exists between metronidazole resistance and infection with TVVs and *M. hominis*. Moreover, we sought to establish whether sensitive and resistant *T. vaginalis* isolates differ in the RNA expression of the *PFOR* gene.

2. Materials and methods

2.1. T. vaginalis isolates

A total of 30 fresh *T. vaginalis* isolates were isolated from urine samples. The parasites were harvested consecutively at Laboratório de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFRGS, Brazil, and were registered and stored by cryopreservation at −80 °C in the *T. vaginalis* isolates bank of our research team. Two isolates, *T. vaginalis* 30236 and 30238, from the American Type Culture Collection (ATCC) were also used. The virus-harboring isolate *T. vaginalis* 347 was used as a positive control in the TVV identification assay. The *M. hominis*-positive isolates were confirmed by 16S rRNA sequencing.

2.2. T. vaginalis isolation and in vitro culture

First, the urine samples were examined by direct microscopy and subsequently inoculated onto trypticase-yeast extract-maltose (TYM) medium (pH 6.0) supplemented with heat-inactivated bovine serum (HIBS 10% [v/v]) and incubated at 37 °C (±0.5), as previously described (Diamond, 1957). The urine samples were considered *T. vaginalis* negative after 5 days of culture. The positive samples were subcultured until axenization. Thereafter, all experiments were performed using organisms in the logarithmic phase of growth that exhibited motility and normal morphology, and all experiments were performed in triplicate and with at least three independent assays.

2.3. Metronidaloze-susceptibility assay

Metronidazole susceptibility was assessed separately for each isolate in this study after log growth was achieved. Briefly, a stock solution (9.35 mM) of metronidazole (Sigma) was prepared in DMSO (4.0%) and TYM medium. Trichomonads were washed three times with glucose (0.2%) saline solution and the concentration was adjusted (5.0×10^4 trophozoites per well) and added to 12 serial dilutions (2,334–1.14 μM) of metronidazole in 96-well plates. Plates were incubated at 37 °C for 48 h under microaerophilic conditions (5% CO_2). After incubation, the plates were examined using an inverted microscope to determine the MIC (minimum inhibitory concentration), the lowest drug concentration at which no motile trichomonads were observed at the end of the incubation period. Low-level resistance was defined as an anaerobic MIC 30–60 μM, moderate-level resistance as 60–120 μM, and high-level resistance as 235 μM or greater as previously described by Butler et al. (2010). The end point was confirmed by the failure of non-motile parasites to grow after re-inoculation into drug-free medium (Rabiee et al., 2012). All experiments were carried out thrice, in triplicate, and with a vehicle control (DMSO).

2.4. Detection of T. vaginalis isolates infected by M. hominis

2.4.1. Polymerase chain reaction (PCR) amplification of 16S rRNA M. hominis

The 16S rRNA *M. hominis* gene was detected by PCR amplification using primers previously described Blanchard et al. (1993): forward 5′ CAATGGCTAATGCCGGATACGC 3′ and reverse 5′GGTACCGTCAGTCTGCAAT 3′. Total DNA was extracted from *T. vaginalis* according to an adaptation of the method described in previous study Butler et al. (2010). Briefly, the DNA was extracted from 4.0×10^6 trophozoites using a boiling based method: the trichomonads were washed three times using glucose (0.2%) saline solution and the pellet was resuspended in 200 μL of TE (10 mM Tris 1.0 mM EDTA pH 8.0) buffer, boiled at 100 °C for 20 min, and centrifuged at 10.000×*g* for 10 min. The supernatant containing the DNA was stored at −20 °C and subsequently used in PCR assays.

Primers specific to the *T. vaginalis* 18S rRNA was used as an internal reaction control: forward 5′ TAATGGCAGAATCTTTGGAG 3′, reverse 5′ GAACTTTAACCGAAGGACTTC 3′ (Blanchard et al., 1993). Parallel reactions performed without DNA were included as negative controls. Amplicons were analyzed on a 1.0% agarose gel in Tris–Borate–EDTA (TBE) buffer containing ethidium bromide (0.5 μg/mL) and photographed under UV visualization. The *M. hominis*-specific and *T. vaginalis*-specific products were 334 and 312 bp, respectively. The negative results were confirmed in three independent experiments.

2.4.2. Identification of 16S rRNA M. hominis by sequencing

The PCR product was purified using the PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen™) for identification of 16S rRNA *M. hominis* by sequencing. Samples were sequenced by ACTGene Análises Moleculares Ltda. (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using an ABI-PRISM 3100 Genetic Analyzer armed with 50 cm capillaries

and POP6 polymer (Applied Biosystems). DNA templates (10 ng) were labeled with 2.5 pmol of each primer (forward 5′ CAATGGCTAATGCCGGATACGC 3′ and reverse 5′GGTACCGTCAGTC TGCAAT 3′) and 3 μL of BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems) in a final volume of 10 μL. Labeling reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems) thermocycler with an initial denaturing step of 96 °C for 3 min, followed by 25 cycles of 96 °C for 10 s, 55 °C for 5 s and 60 °C for 4 min. Labeled samples were purified by isopropanol precipitation followed by rinsing with 70% ethanol. Precipitated products were suspended in 10 μL formamide, denatured at 95 °C for 5 min, ice-cooled for 5 min and electroinjected in the automatic sequencer. The similarity of nucleotides sequences were evaluated using Basic Local Alignment Search Tool (BLAST) and the 16S rRNA *M. hominis* sequence alignment was made using ClustalW – Multiple Sequence Alignment (EBI, United Kingdom).

2.5. Detection of *T. vaginalis* harboring TVV and identification of TVV species

2.5.1. dsRNA extraction

All 33 isolates were screened and evaluated for the presence of TVVs by dsRNA extraction. The dsRNA were extracted from 1.0×10^7 trophozoites using adaptations of protocols previously described (Attoui et al., 2000; Goodman et al., 2011a). Trichomonads were washed three times using glucose (0.2%) saline solution (pH 8.0) and the total RNA was subsequently extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Next, contaminating single-stranded RNA was selectively precipitated by incubation with 4.0 M LiCl at 4 °C overnight and centrifuged at 12.750×*g* for 5 min. The dsRNA was precipitated by the addition of isopropanol and ammonium acetate (7.5 M) and incubation at −20 °C for 2 h. Then, the samples were centrifuged at 12.750×*g* for 5 min and the pellets were resuspended in ethanol (75%) and centrifuged at 7.750×*g* for 5 min. The pellets were dissolved in water to create a working solution of dsRNA. Visualization of dsRNA was performed with ethidium bromide (0.5 μg/mL) under UV transillumination after electrophoresis on 1.0% agarose gels. The presence of a band between 4 kbp and 5 kbp was positive for *Trichomonasvirus* (TVVs) (Goodman et al., 2011a).

2.5.2. TVV species identification by reverse transcriptase polymerase chain reaction (RT-PCR)

All TVV-positive *T. vaginalis* isolates in the dsRNA initial screening were identified at the species level by an RT-PCR assay using species-specific primers (Table 1) (Goodman et al., 2011a). All primers used amplified a 500 pb product. Briefly, the dsRNA extracts from cultured *T. vaginalis* organisms were used to carry out RT-PCR using the Qiagen one-step RT-PCR kit (Qiagen®) according to the manufacturer's instructions. Negative control was carried out with no RNA template. The negative results were confirmed in three independent experiments.

Table 1
TVVs species-specific primers for RT-PCR.

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

2.6. Relative RNA expressions levels of pyruvate-ferredoxin oxidoreductase (PFOR)

To evaluate the possible role of *PFOR* in the metronidazole resistance mechanism, the relative RNA expression levels of this gene were evaluated in the metronidazole-sensitive *T. vaginalis* isolates and those with low and moderate resistance levels. The trophozoites were treated for 24 h with a low concentration of metronidazole (2 μM) before RNA extraction, and the trophozoites cultivated without MTZ were used as the control. The *PFOR* primer pair used, was designed for our previous study (unpublished data): Forward 5′CTCGTTTGGGGTGCTACATT3′ and Reverse 5′TCCTGATC CCAAACCTTGAG3′ (TVAG_198110; 239 bp). The total RNA was extracted from $\sim 4.0 \times 10^6$ trophozoites/mL using TriZol™ following the manufacturer's instructions and stored −80 °C until use. Five 10-fold series dilutions starting from 100 ng of genomic DNA was used to construct a standard curves in order to determine PCR amplification efficiencies, and all PCR efficiencies were greater than 95%. Quantitative real-time PCR reactions (qRT-PCR) were carried out in 0.1-mL microtubes using Qiagen's real-time PCR system, Rotor-Gene Q and the Rotor-Gene™ SYBR™ Green RT-PCR kit (Qiagen™). Each PCR reaction contained 6 μL of 2× of Rotor-Gene SYBR Green PCR Master Mix, 200 nM of each primer, 0.125 μL of Rotor-Gene RT Mix and 100 ng of RNA template in a total volume of 12 μL. The cycling conditions were as follows: an initial step at 55 °C for 10 min for reverse transcription; an enzyme activation step at 95 °C for 10 min; 35 cycles of denaturation at 95 °C for 15 s; and annealing and extension at 64 °C for 30 s, with fluorescence data collection in this step. Melting curve analyses were performed by raising the temperature at the end of each run in by 1 °C per 5 s from 63 °C or 64 °C to 95 °C. Parallels reactions without RNA or DNA templates were run as negative controls. Three biological replicate samples were analyzed in three technical replicates under each experimental condition.

The relative change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The levels of *PFOR* mRNA were quantified by qRT-PCR analysis relative to *α-tubulin* and *actin* mRNA as reference genes, as previously determined by us (unpublished data). Three technical replicates from three biological replicates were analyzed.

2.7. Statistical analysis

The existence of an association between the MIC value for metronidazole and the presence or absence of *M. hominis* and TVVs was analyzed using a X^2 test following residual analysis. All statistical analyses were performed in SPSS 18.0 (SPSS Inc., Chicago, IL, USA), and a p value <0.05 was considered statistically significant. The statistical significance of the RNA expression changes of *PFOR* between sensitive and resistant *T. vaginalis* isolates were calculated using one-way ANOVA. The level of significance was also determined by the Bonferroni method comparing all groups versus the control ($P < 0.05$).

2.8. Ethical aspects

This study was submitted and approved by the UFRGS Ethical Committee, number 18923.

3. Results

3.1. *T. vaginalis* isolation

Five hundred and thirty urine samples were collected in a survey spanning a two-year period (2011–2012). Overall, 30 out of

530 (5.7%) urine samples were positive for *T. vaginalis* infection. One long-term grown *T. vaginalis* isolate cultivated at the laboratory (347 isolate, from the USA) and two ATCC isolates (30236 and 30238) were included in this study for a total enrollment of thirty-three *T. vaginalis* isolates. Considering only the fresh *T. vaginalis* isolates, a total of 4.5% (24 out of 530) were isolated from female patients and 1.1% (6 out of 530) were isolated from male patients. The minimum and maximum of age among women with trichomoniasis was 15 and 74 years and the mean of age was 49.9 ± 21.4 SD years. The highest infection rate occurred among younger women: 41.7% were less than 25 years old. In addition, a high rate of infection was observed among older women (>60 years old), accounting for 33% of the T. vaginalis-infected women. The mean age of T. vaginalis-infected men was 69.8 ± 19.9 SD years, which ranged from 41 to 87 years.

3.2. T. vaginalis isolates were infected by M. hominis

The presence of *M. hominis* was detected in 56.7% (17 out of 30) fresh clinical *T. vaginalis* isolates (Table 2). Taking into consideration only the fresh *T. vaginalis* isolates, the prevalence of *M. hominis* infection was 62.5% (15 out of 24) among female patients, whereas among male patients, the prevalence was lower at 33.3% (2 out of 6). The long-term grown cultivated isolate (347) and the ATCC isolates (30236 and 30238) were also *M. hominis*-infected. The PCR amplification for each *T. vaginalis* isolate was assessed by agarose gel electrophoresis and the *M. hominis* infection was confirmed by the presence of a 320 pb amplification product (data not shown). All 20 positive isolates for 16S RNA *M. hominis* in the PCR reactions were also correctly identified as *M. hominis* by 16S RNA gene sequencing. The similarities values ranged from 98% to 100%. The alignment of four *M. hominis*-positive *T. vaginalis* isolates is shown in Fig. 1 and Supplemental Figure 1.

Table 2
MIC values for metronidazole and *Trichomonas vaginalis* isolates infected by *Mycoplasma hominis* and TVV species.

Long-term grown isolates	MIC (μM)	*M. hominis*	TVV1	TVV2	TVV3	TVV4
ATCC236	1.14	+	+	–	+	–
ATCC238	9.12	+	–	–	+	–
347	4.56	+	+	–	–	–
Fresh clinical isolates						
TV-LACM1	9.12	–	+	–	–	–
TV-LACM2	18.25	+	–	–	+	–
TV-LACM3	9.13	–	+	–	–	–
TV-LACM4	9.13	–	+	–	–	–
TV-LACM5	4.56	+	+	–	–	–
TV-LACM6	18.25	+	+	–	–	–
TV-LACM7	18.25	+	+	–	–	–
TV-LACM8	9.13	+	+	+	–	–
TV-LACM9	4.56	+	+	–	–	–
TV-LACM10	9.13	–	+	–	–	–
TV-LACM11	9.13	+	–	+	+	+
TV-LACM12	18.25	+	+	–	+	–
TV-LACM13	4.56	+	+	+	–	–
TV-LACM14	36.50	+	–	–	–	–
TV-LACM15	73.00	–	–	–	–	–
TV-LACM16	4.56	+	–	+	+	–
TV-LACM17	18.25	+	+	+	+	–
TV-LACM18	4.56	–	+	–	–	–
TV-LACM19	36.50	–	–	+	–	–
TV-LACM20	9.13	+	+	–	–	+
TV-LACM21	4.56	–	–	+	–	–
TV-LACM22	18.25	+	–	–	–	–
TV-LACM23	36.50	–	–	–	+	–
TV-LACM24	18.25	+	+	+	+	–
TV-LACH1	4.56	+	+	–	–	–
TV-LACH2	18.25	–	+	–	–	–
TV-LACH3	9.13	–	+	–	–	–
TV-LACH4	9.13	–	+	+	+	+
TV-LACH5	9.13	–	+	–	–	–
TV-LACH6	9.13	+	+	–	+	–

3.3. High rates of T. vaginalis isolates harboring TVV species

We found a high number of TVV-positive *T. vaginalis* isolates by dsRNA initial screening and by RT-PCR assay for identification at the species level; all given a specific 500 pb amplification product (data not shown). Regarding the presence of TVVs, 90% (27 out of 30) of the *T. vaginalis* isolates harbored viruses (Table 2). Among these, 33.3% (10 out of 30) were infected with more than one TVV species. TVV1 was the most prevalent species (77.5%), followed by TVV2 and TVV3 (30%). The least frequent species was TVV4 (10%) (Table 2). The prevalence of TVV infection among *T. vaginalis* isolated from female patients was 87.2% (21 out of 24) while among isolates from male patients, the prevalence of TVV-infection was 100% (6 of 6).

3.4. T. vaginalis isolates metronidazole susceptibility

The results of the *in vitro* metronidazole susceptibility experiments are described in Table 2. Most of the isolates 86.7% (26 of 30) evaluated in this study were metronidazole sensitive, with MIC values ranging from between 1.14 μM to 18.25 μM. Of the 30 *T. vaginalis* fresh isolates, four (13.3%) were metronidazole resistant. The TV-LACM14, TV-LACM19 and TV-LACM23 isolates presented MIC values of 36.5 μM and therefore had low-level resistance. The TV-LACM15 isolate had moderate-level resistance with a MIC value of 73 μM. The statistical analysis showed no association between both TVV and *M. hominis* infection ($P = 0.245$ and 0.24, respectively) and metronidazole resistance in *T. vaginalis*.

3.5. PFOR RNA expression is not the main mechanism of resistance to metronidazole

The relative RNA expression levels of *PFOR* from four *T. vaginalis* isolates with different metronidazole resistance profiles are described in Fig. 2. The mRNA levels of *PFOR* were lowest in both isolates of TV-LACM15, which had moderate-level metronidazole resistance, and in TV-LACM14, which had low-level metronidazole resistance. The expression level of *PFOR* mRNA was highest in TV-LACM19, which had low-level metronidazole resistance, and in ATCC 30238, which is metronidazole-sensitive.

4. Discussion

Metronidazole and tinidazole are nitroimidazoles and are the drugs of choice recommended by the Food and Drug Administration (FDA, USA) for the treatment of human trichomoniasis (Helms et al., 2008). The prevalence of resistant isolates seems to vary widely, ranging from 2.2% to 9.6% (Pérez et al., 2001; Schwebke and Barrientes, 2006). Increasing reports of failures in the treatment of *T. vaginalis* infection and metronidazole-resistant *T. vaginalis* isolates have occurred over recent years, and evidence suggests that resistance may be increasing (Miller and Nyirjesy, 2011). In our findings, 13.3% of the fresh *T. vaginalis* isolates were metronidazole resistant, although none of them had high levels of resistance. The TV-LACM14, TV-LACM19 and isolates had low resistance whereas the TV-LACM15 isolate was moderately resistant. These results are similar to results obtained in a previous surveillance study in the US, which found that 4.3% of 538 *T. vaginalis* isolates exhibited low-level *in vitro* metronidazole resistance. However, in that same study, *T. vaginalis* with moderate or high-level resistance were not found (Kirkcaldy

```
CLUSTAL 2.1 Multiple Sequence Alignment

ATCC30238       TTCATCGTGCACGCTGTGTCGCTCCATCAAGCTTTCGCTCATTGTGGAATATTCCCTACT
TVLACM14        TTCATCGTGCACGCTGTGTCGCTCCATCAAGCTTTCGCTCATTGTGGAATATTCCCTACT
TVLACM11        TTCATCGTGCACGCTGTGTCGCTCCATCAAGCTTTCGCTCATTGTGGAATATTCCCTACT
TVLACH6         TTCATCGTGCACGCTGTGTCGCTCGATCAAGCTTTCGCTCATTGTGGAATATTCCCTACT
                ********************** ***********************************

ATCC30238       GCTGCCTCCCGTAGGAGTTTGGGCCGTATCTCAGTCCCAATGTGGCCGTTCAGTCTCTCG
TVLACM14        GCTGCCTCCCGTAGGAGTTTGGGCCGTATCTCAGTCCCAATGTGGCCGTTCAGTCTCTCG
TVLACM11        GCTGCCTCCCGTAGGAGTTTGGGCCGTATCTCAGTCCCAATGTGGCCGTTCAGTCTCTCG
TVLACH6         GCTGCCTCCCGTAGGAGTTTGGGCCGTATCTCAGTCCCAATGTGGCCGTTCAGTCTCTCG
                ************************************************************

ATCC30238       ACCCGGCTAAACATCATAGTCTTGGTGGGCCATTACCTCACCAACTAACTAATGTTCCGC
TVLACM14        ACCCGGCTAAACATCATAGTCTTGGTGGGCCATTACCTCACCAACTAACTAATGTTCCGC
TVLACM11        ACCCGGCTAAACATCATAGTCTTGGTGGGCCATTACCTCACCAACTAACTAATGTTCCGC
TVLACH6         ACCCGGCTAAACATCATAGTCTTGGTGGGCCATTACCTCACCAACTAACTAATGTTCCGC
                ************************************************************

ATCC30238       ACCCTCATCTTTTAGTGGCGCCTTACAGCGCCTTTCACAACGGAACCATGCGGTTCCATG
TVLACM14        ACCCTCATCTTTTAGTGGCGCCTTACAGCGCCTTTCACAACGGAACCATGCGGTTCCATG
TVLACM11        ACCCTCATCTTTTAGTGGCGCCTTACAGCGCCTTTCACAACGGAACCATGCGGTTCCATG
TVLACH6         ACCCTCATCTTTTAGTGGCGCCTTACAGCGCCTTTCACAACGGAACCATGCGGTTCCATG
                ************************************************************

ATCC30238       CGTATCCGGCATTAGCCATTGA
TVLACM14        CGTATCCGGCATTAGCCATTGA
TVLACM11        CGTATCCGGCATTAGCCATTGA
TVLACH6         CGTATCCGGCATTAGCCATTGA
                **********************
```

Fig. 1. Multiple 16s RNA *Mycoplasma hominis* sequence alignment obtained by ClustalW. The sequences are representative of control, ATCC 30238 (metronidazole-sensitive), and fresh clinical isolates: TV-LACM14, low-level resistance and no TVV infection; TV-LACM11, metronidazole-sensitive, harboring TVV2, TVV3 and TVV4; TV-LACH6, metronidazole-sensitive, harboring TVV1 and TVV3.

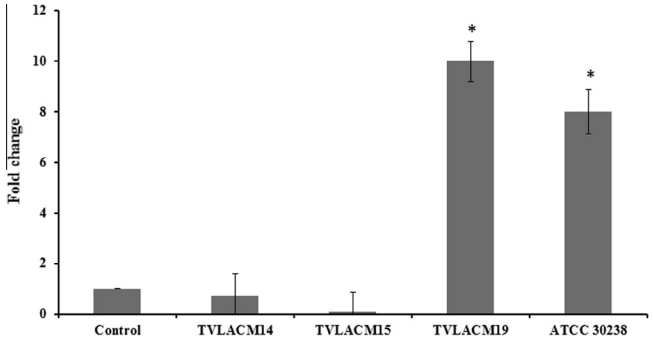

Fig. 2. Relative *PFOR* RNA expression of four *T. vaginalis* isolates with different metronidazole sensitivity profiles. TV-LACM15, moderate metronidazole resistance; TV-LACM14 and TV-LACM19, low metronidazole resistance; ATCC30236 metronidazole sensitive. The bars and errors bars represent the value and standard deviation from the relative RNA expression of *PFOR* obtained from three independent experiments performed in triplicate. (*) indicates a significant difference.

et al., 2012). By contrast, a previous prevalence study (Schwebke and Barrientes, 2006) found 17.6% of *T. vaginalis* isolates had moderate to high metronidazole resistance levels. Considering these results from different regions of the world may highlight the possibility that reliance on a single class of antimicrobial drugs for treating *T. vaginalis* infections may heighten the vulnerability to the emergence of resistance. Consequently, novel treatment options are needed.

Trichomoniasis is characterized by significant heterogeneity in its clinical presentation, metronidazole sensitivity and its contribution to increasing the host susceptibility to other infectious agents. However, it is not clear whether this clinical variability is more influenced by host factors or by differences in the phenotypic expression of individual *T. vaginalis* isolates (Meade et al., 2009). Therefore, after considering age and sex in characterizing patients with trichomoniasis profiles, our concern was the molecular epidemiology among *T. vaginalis* isolates. In support of our question and to our best knowledge, there is no molecular epidemiology data from *T. vaginalis* isolates from any region of Brazil. We also attempted to understand whether there is any association between metronidazole resistance and molecular aspects, such as the presence of *M. hominis* and TVV, in fresh clinical *T. vaginalis* isolates. Thirty-three isolates from female and male patients, including both fresh and ATCC organisms, were tested for their susceptibility to metronidazole and the presence of *M. hominis* and TVVs.

The ability of *M. hominis* to invade, survive and multiply in the cytoplasm of *T. vaginalis* is an important defense mechanism of these bacteria during human infection because the parasite plays a "Trojan horse" role (Dessì et al., 2005, 2006) by providing a protected niche for the survival of the bacterium. Our study is the first report employing sequencing methodology to certify this analysis and we found that 56.7% of fresh *T. vaginalis* isolates were infected with *M. hominis*. This result corroborates with the study performed in 28 *T. vaginalis* isolates from China, which found that 50% of the parasites were infected by this bacterium (Xiao et al., 2006). However, the prevalence described in the literature varies widely. A study was conducted with 40 isolates from different geographical regions (Italy, Angola, and Mozambique), and 37 (92.5%) were infected with *M. hominis* (Rappelli et al., 2001). In contrast, other studies found a lower prevalence of infected isolates (20% and 25%, respectively) (Butler et al., 2010; Hampl et al., 2001). The presence of *M. hominis* in association with *T. vaginalis* has important clinical implications and this symbiosis has been correlated with the metronidazole resistance of the parasite (Xiao et al., 2006). However, the present study revealed a high incidence of *T. vaginalis* isolates infected by *M. hominis*, most of which were susceptible to metronidazole. These important findings confirm the results that showed no association between metronidazole resistance and *M. hominis* infection (Butler et al., 2010) and do not support the hypothesis that the presence of *M. hominis* is sufficient to

confer metronidazole resistance to *T. vaginalis* isolates (Xiao et al., 2006).

Approximately one half of all *T. vaginalis* isolates are infected with dsRNA or VLP viruses (Gerhold et al., 2009). However, a large variability is observed in the prevalence of this infection. Our study found that 90% of the fresh isolates were infected with TVVs, confirming the high prevalence observed in previously study that found dsRNA particles in 82% of *T. vaginalis* isolates in South Africa (Weber et al., 2003). A study conducted in Korea found that only 13.6% of the isolates were infected with TVVs, showing that the infection rate may vary according to geographical location (Kim et al., 2007). Regarding the prevalence of TVVs species, TVV1 was found to be the most prevalent, followed by TVV2, TVV3, and TVV4. Five *T. vaginalis* isolates were investigated and also found that TVV1 was the most prevalent species Goodman et al. (2011a). In contrast, other researchers (Fraga et al., 2012) considered only TVV1 and TVV2 species and found a higher prevalence of TVV2 (Xiao et al., 2006) relative to TVV1 (Helms et al., 2008) among 21 *T. vaginalis* isolates. Three of their isolates were co-infected with both species (14.3%). As 10 of 30 (33.3%) infected isolates showed more than one TVV species, the prevalence of co-infection was higher in our study.

The association between the presence of TVVs and metronidazole resistance in T. vaginalis has been suggested elsewhere (Snipes et al., 2000; Hampl et al., 2001). However, our findings clearly demonstrate the lack of relationship between the presence of TVVs and metronidazole resistance because 90% of the isolates were infected with TVVs and most of them were metronidazole-sensitive. Furthermore and importantly, TV-LACM15 was the only moderately metronidazole-resistant isolate, but was TVVs- and *M. hominis*-free. Though the mechanism of metronidazole resistance in *T. vaginalis* is not fully understood, the most accepted hypothesis is that resistance is achieved through a decrease or loss of ferredoxin (Fdx) and pyruvate ferredoxin oxidoreductase (PFOR) enzymatic activity involved in drug activation (Rasoloson et al., 2002). Moreover, metronidazole resistance involves the loss of multiple hydrogenosomal proteins, including Fdx, PFOR, malic enzyme (ME), and hydrogenase (Land et al., 2004). Our findings do not support the role of *PFOR* RNA expression as the main mechanism of resistance to metronidazole. Although *PFOR* RNA expression was down-regulated in TV-LACM15 and TV-LACM14 isolates, which had moderate and low resistance levels, respectively, the *PFOR* RNA expression level in the TV-LACM19 isolate with low level resistance was higher than in ATCC30238, which is a metronidazole-sensitive isolate.

The complete *T. vaginalis* genome sequence published by Carlton et al. (2007) is a good starting point and greatly facilitates basic research in molecular biology, particularly for metronidazole resistance. Thus, it could easily lead to the identification of genetic markers for resistance and development of molecular tests, although the genome available is from metronidazole sensitive isolate. Nowadays, the complete understanding of metronidazole resistance has been challenged because of the size, complexity, and repetitiveness of the *T. vaginalis* genome. For example, other possible mechanisms were identified, along with the presence of nitroreductase *NimA-like* genes, which have been implicated in metronidazole resistance in bacteria. In addition, a recent study identified single nucleotide polymorphisms (SNPs) in two nitroreductase genes (*ntr4Tv* and *ntr6Tv*) associated with resistance (Paulish-Miller et al., 2014). Consequently, further molecular studies are necessary to establish the exact mechanism of drug resistance in *T. vaginalis*.

Overall, our findings demonstrated a high prevalence of *T. vaginalis* isolates infected with both *M. hominis* and different TVVs species. Moreover, a major metronidazole-sensitivity profile was also observed. Thus, our data strongly suggest that a symbiotic relationship between these bacteria, the viruses and the protozoa is not associated with drug resistance. Our current efforts are aimed at addressing whether the presence of *M. hominis* and TVVs may modulate the pathogenesis of *T. vaginalis* isolated from Brazil.

Acknowledgments

Special acknowledgement is given to Dr. Marlene Benchimol (Universidade Santa Úrsula, RJ, Brazil) for the kind donation of the 347 *T. vaginalis* isolate. This work was supported by the NANOBIOTEC-Brazil program from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil (Grant 23038.019022/2009-68) to A.J.M. and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil (Grants 475315/2011-1 and 474930/2012-2) to T.T. D.L.B., O.S. and A.P.F. are recipient of scholarships from CAPES/Brazil, T.T. and A.J.M. thank CNPq for researcher fellowships.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.07.005.

References

Attoui, H., Frédérique, B., Cantaloube, J.F., Biagini, P., Micco, P., et al., 2000. Strategies for the sequence determination of viral dsRNA genomes. J. Virol. Methods 89, 147–1458.

Benchimol, M., Chang, T.H., Alderete, J.F., 2002. Visualization of new virus-like-particles in *Trichomonas vaginalis*. Tissue Cell 34, 406–415.

Blanchard, A., Yanez, T.A., Dybvig, K., Watson, H.L., Griffiths, G., 1993. Evaluation of intraspecies genetic variation within the 16S rRNA gene of *Mycoplasma hominis* and detection by polymerase chain reaction. J. Clin. Microbiol. 31, 1358–1361.

Butler, S.E., Augostini, P., Secor, W.E., 2010. *Mycoplasma hominis* infection of *Trichomonas vaginalis* is not associated with metronidazole-resistant trichomoniasis in clinical isolates from the United States. Parasitol. Res. 107, 1023–1027.

Carlton, J.M., Hirt, R.P., Silva, J.C., Delcher, A.L., Schatz, M., et al., 2007. Draft genome sequence of the sexually transmitted pathogen Trichomonas vaginalis. Science 315, 207–212.

Dessì, D., Delogu, G., Emonte, E., Catania, M.R., Fiori, P.L., et al., 2005. Long-term survival and intracellular replication of *Mycoplasma hominis* in *Trichomonas vaginalis* cells: potential role of the protozoan in transmitting bacterial infection. Infect. Immun. 73, 1180–1186.

Dessì, D., Rappelli, P., Diaz, N., Cappuccinelli, P., Fiori, P.L., 2006. *Mycoplasma hominis* and *Trichomonas vaginalis*: a unique case of symbiotic relationship between two obligate human parasites. Front. Biosci. 11, 2028–2034.

Diamond, L.S., 1957. The establishment of various *Trichomonas* of animals and man in axenic cultures. J. Parasitol. 43, 488–490.

Fraga, J., Rojas, L., Sariego, I., Fernández-Calienes, A., Angel Nuñez, F., 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.

Gerhold, R.W., Allison, A.B., Sellers, H., Linnemann, E., Chang, T.H., et al., 2009. Examination for double-stranded RNA viruses in *Trichomonas gallinae* and identification of a novel sequence of a *Trichomonas vaginalis* virus. Parasitol. Res. 105, 775–779.

Goodman, R.P., Freret, T.S., Kula, T., Geller, A.M., Talkington, M.W.T., et al., 2011a. Clinical isolates of *Trichomonas vaginalis* concurrently infected by strains of up to four *Trichomonasvirus* species (Family *Totiviridae*). J. Virol. 85, 4258–4270.

Goodman, R.P., Ghabrial, S.A., Fichorova, R.N., Nibert, M.L., 2011b. *Trichomonasvirus*: a new genus of protozoan viruses in the family *Totiviridae*. Arch. Virol. 156, 171–179.

Grodstein, F., Goldman, M.B., Cramer, D.W., 1993. Relation of tubal infertility to a story of sexually transmitted diseases. Am. J. Epidemiol. 137, 577–584.

Hampl, V., Vanacova, S., Kulda, J., Flegr, J., 2001. Concordance between genetic relatedness and phenotypic similarities of *Trichomonas vaginalis* strains. BMC Evol. Biol. 1, 11.

Helms, D.J., Mosure, D.J., Secor, W.E., Workowski, K.A., 2008. Management of *Trichomonas vaginalis* in women with suspected metronidazole hypersensitivity. Am. J. Obstet. Gynecol. 198, 370–377.

Khoshnan, A., Alderete, J.F., 1994. *Trichomonas vaginalis* with a double-stranded RNA virus has upregulated levels of phenotypically variable immunogen mRNA. J. Virol. 68, 4035–4038.

Kim, J.W., Chung, P.R., Hwang, M.K., Choi, E.Y., 2007. Double-stranded RNA virus in Korean isolate IH-2 of *Trichomonas vaginalis*. Korean J. Parasitol. 45, 87–94.

Kirkcaldy, R.D., Augostini, P., Asbel, L.E., Bernstein, K.T., Kerani, R.P., et al., 2012. *Trichomonas vaginalis* antimicrobial drug resistance in 6 US Cities, STD Surveillance Network, 2009–2010. Emerg. Infect. Dis. 18, 939–943.

Kissinger, P., 2015. Epidemiology and treatment of trichomoniasis. Curr. Infect. Dis. Rep. 17, 484.

Klebanoff, M.A., Carey, J.C., Hauth, J.C., Hiller, S.L., Nugent, R.P., et al., 2001. Failure of metronidazole to prevent preterm delivery among pregnant women with asymptomatic *Trichomonas vaginalis* infection. N. Engl. J. Med. 345, 487–493.

Land, K.M., Delgadillo-Correa, M.G., Tachezy, J., Vanacova, S., Hsieh, C.L., et al., 2004. Targeted gene replacement of a ferredoxin gene in *Trichomonas vaginalis* does not lead to metronidazole resistance. Mol. Microbiol. 51, 115–122.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method 25, 402–408.

Meade, J.C., de Mestral, J., Stiles, J.K., Secor, W.E., Finley, R.W., et al., 2009. 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. 80, 245–251.

Miller, M.R., Nyirjesy, P., 2011. Refractory trichomoniasis in HIV-positive and HIV-negative subjects. Curr. Infect. Dis. Rep. 13, 595–603.

Paulish-Miller, T.E., Augostini, P., Schuyler, J.A., Smith, W.L., Mordechai, E., et al., 2014. *Trichomonas vaginalis* metronidazole resistance is associated with single nucleotide polymorphisms in the nitroreductase genes *ntr4Tv* and *ntr6Tv*. Antimicrob. Agents Chemother. 58, 2938–2943.

Pérez, S., Fernández-Verdugo, A., Pérez, F., Vázquez, F., 2001. Prevalence of 5-nitroimidazole-resistant *Trichomonas vaginalis* in Oviedo, Spain. Sex. Transm. Dis. 28, 115–116.

Rabiee, S., Bazmani, A., Matini, M., Fallah, M., 2012. Comparison of resistant and susceptible strains of *Trichomonas vaginalis* to metronidazole using PCR method. Iran. J. Parasitol. 7, 24–30.

Rappelli, P., Addis, M.F., Carta, F., Fiori, P.L., 1998. *Mycoplasma hominis* parasitism of *Trichomonas vaginalis*. Lancet 352, 1286.

Rappelli, P., Franco, C., Giuseppe, D., Addis, M.F., Dessì, D., et al., 2001. *Mycoplasma hominis* and *Trichomonas vaginalis* symbiosis: multiplicity of infection and transmissibility of *M. hominis* to human cells. Arch. Microbiol. 175, 70–74.

Rasoloson, D., Vanacová, S., Tomková, E., Rázga, J., Hrdý, I., et al., 2002. Mechanisms of *in vitro* development of resistance to metronidazole in *Trichomonas vaginalis*. Microbiology 148, 2467–2477.

Schwebke, J.R., Barrientes, F.J., 2006. Prevalence of *Trichomonas vaginalis* isolates with resistance to metronidazole and tinidazole. Antimicrob. Agents Chemother. 50, 4209–4210.

Snipes, L.J., Gamard, P.M., Narcisi, E.M., Beard, C.B., Lehmann, T., et al., 2000. Molecular epidemiology of metronidazole resistance in a population of *Trichomonas vaginalis* clinical isolates. J. Clin. Microbiol. 38, 3004–3009.

Stark, J.R., Judson, G., Alderete, J.F., Mundodi, V., Kucknoor, A.S., et al., 2009. Prospective study of *Trichomonas vaginalis* infection and prostate cancer incidence and mortality: Physicians' Health Study. J. Natl. Cancer Inst. 101, 1406–1411.

Sutcliffe, S., Giovannucci, E., Alderete, J.F., Chang, T.H., Gaydos, C.A., et al., 2006. Plasma antibodies against *Trichomonas vaginalis* and subsequent risk of prostate cancer. Cancer Epidemiol. Biomarkers Prev. 15, 939–945.

Two, O., Dessí, D., Vu, A., Mercer, F., Stevens, G.C., et al., 2014. *Trichomonas vaginalis* homolog of macrophage migration inhibitory factor induces prostate cell growth, invasiveness, and inflammatory responses. PNAS 111, 8178–8179.

Viikki, M., Pukkala, E., Nieminen, P., Hakama, M., 2000. Gynaecological infections as risk determinants of subsequent cervical neoplasia. Acta Oncol. 39, 71–75.

Wang, A.L., Wang, C.C., 1985. A linear double-stranded RNA in *Trichomonas vaginalis*. J. Biol. Chem. 260, 3697–3702.

Weber, B., Mapeka, T.M., Maahlo, M.A., Hoosen, A.A., 2003. Double stranded RNA virus in South African *Trichomonas vaginalis* isolates. J. Clin. Pathol. 56, 542–543.

Wendel, K., Rompalo, A., Erbelding, E., Chang, T.H., Alderete, J.F., 2002. Double-stranded RNA viral infection of *Trichomonas vaginalis* infecting patients attending a sexually transmitted diseases clinic. J. Infect. Dis. 186, 558–561.

WHO – World Health Organization, 2012. Global Incidence and Prevalence of Selected Curable Sexually Transmitted Infections – 2008. World Health Organization.

Xiao, J.C., Xie, L.F., Fang, S.L., Gao, M.Y., Zhu, Y., et al., 2006. Symbiosis of *Mycoplasma hominis* in *Trichomonas vaginalis* may link metronidazole resistance *in vitro*. Parasitol. Res. 100, 123–130.