Prevalence of double-stranded RNA virus in Trichomonas vaginalis isolated in Italy and association with the symbiont Mycoplasma hominis

Prevalence of double-stranded RNA virus in *Trichomonas vaginalis* isolated in Italy and association with the symbiont Mycoplasma hominis

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Abstract

The flagellated protozoon Trichomonas vaginalis, responsible for trichomoniasis, can establish a symbiotic relationship with the bacterium Mycoplasma hominis and can harbor double-stranded RNA Trichomonasvirus (TVV). In this study, we investigated by real-time PCR the prevalence of the four TVVs and of *M. hominis* among 48 *T. vaginalis* strains isolated in Italy, and we evaluated a possible association with metronidazole resistance. Fifty percent of the analyzed trichomonad strains tested positive for at least one TVV *T. vaginalis*, with TVV2 being the most prevalent, followed by TVV1 and TVV3. Two *T. vaginalis* strains were infected by TVV4, detected in Europe for the first time. Interestingly, we found more than one TVV species in 75% of positive trichomonad strains. *M. hominis* was present in 81.25% of *T. vaginalis* isolates tested, and no statistically significant association was observed with the infection by any TVV. Metronidazole sensitivity of *T. vaginalis* isolates was evaluated in vitro, and no correlation was observed between minimal lethal concentration and the presence of TVVs. This is the first report on TVV infection of *T. vaginalis* in Italy. Even if no association of TVV positive isolates with the presence of the symbiont *M. hominis* or with metronidazole resistance was observed, further studies are needed to shed light on the effective role of infecting microorganisms on the pathophysiology of *T. vaginalis*.

Keywords Trichomonasvaginalis . Double-stranded RNAvirus . Mycoplasma hominis . Metronidazoleresistance . Symbiosis Real-time PCR

Introduction

Trichomonas vaginalis, a flagellated protozoon colonizing the mucosal epithelium of human urogenital tract, is the etiological agent of trichomoniasis. The infection in women leads to vaginitis, urethritis, and gyneco-obstetric complications, including low birth weight and premature delivery in women

(Hobbs et al. 2008; Thi Trung Thu et al. 2018). In men, trichomoniasis occurs mainly without overt symptoms, and infection has been recently associated with aggressive prostate cancers (Twu et al. 2014). Trichomonas vaginalis is able to establish a

strong clinical association with Mycoplasma hominis, an obligate human parasite colonizing the lower urogenital tract (Dessì et al. 2005). Since 1998, when our group identified for the first time viable mycoplasmas in *T. vaginalis* clinical isolates (Rappelli et al. 1998), several groups have confirmed the presence of *M. hominis* in trichomonad isolates of different geographic origin, with infection rates ranging from 5 to over 89% (Fichorova et al. 2017). It has been demonstrated that the presence of *M. hominis* can influence the physiopathogenicity of the protozoon (Margarita et al. 2016). Interestingly, the protozoon can also harbor up to four linear double-stranded RNA (dsRNA) viruses, ranging in size from 4.5 to 5 Kbp. These viruses, named Trichomonas vaginalis virus (TVV1, TVV2, TVV3, and TVV4), have been recently classified by the International Committee on Taxonomy of Viruses as Trichomonasvirus genus within the Totiviridae family, that includes viruses infecting other protozoa, as Giardiavirus and Leishmaniavirus (Goodman et al. 2011). TVVs lack the virion-associated machinery to infect new trichomonad cells and are transmitted during binary fission (Goodman et al. 2011). It has been demonstrated that more than one TVV species can coexist in the same *T. vaginalis* cell (Benchimol et al. 2002). Recent studies have reported high infection rates ofTrichomonasvirus indifferent protozoan isolates around the globe, ranging from 40 to 100%(Fichorova et al. 2017). TVV1 and TVV2 are the most prevalent viral species identified in *T. vaginalis* isolates, followed by TVV3 and TVV4 (Goodman et al. 2011). In Europe, the only epidemiological study on the prevalence of the different four TVV species in *T. vaginalis* isolates was carried out by Jehee and colleague in the Nederlands (Jehee et al. 2017). They demonstrated that the 50.4% of *T. vaginalis* isolates tested harbored at least one TVV species. The most common virus in this group was TVV1, followed by TVV2 and TVV3, while TVV4 was not detected.

The presence of TVVs may influence the symptomatology of trichomoniasis, increasing the severity of vaginal discharge, dysuria, and erythema (Fichorova et al. 2017). In fact, several in vitro studies have shown that TVVs may modulate the virulence of *T. vaginalis* by modifying parasite gene expression and stimulating pro-inflammatory innate responses (Fichorova et al. 2012). The presence of viruses seems to influence also the growth kinetics of *T. vaginalis*: it was observed that *T. vaginalis* strains infected by TVVs were harder to maintain in culture compared with uninfected strains (Rivera et al. 2017). Currently, the 5-nitroimidazole derivatives metronidazole (MTZ) is the most effective drug for trichomoniasis treatment, but resistance is constantly rising (Küng et al. 2019). It is not clear if the presence of TVVs can influence the resistance of *T. vaginalis*: some studies suggest a correlation between drug resistance and TVVinfection, while others report a lack of connection (Graves et al. 2019a, b).

The objectives of this study were to examine the prevalence of TVV in *T. vaginalis* isolated in Italy, and to examine the association of TVV-positive isolates with MTZ sensitivity and with the presence of the symbiont *M. hominis*.

Materials and methods

Trichomonas vaginalis culture

A total of 48 *T. vaginalis* clinical samples were isolated from 1994 to 2017 Sassari (Italy) from women affected by acute trichomoniasis. The cells were cultured in Diamond's TYM supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere (Dessì et al. 2005) and stored in liquid nitrogen until used.

Nucleic acid extraction and cDNA synthesis

Exponentially growing cells from 48 *T. vaginalis* strains were subjected to RNA and DNA extraction. Genomic DNAs were extracted by commercial kit DNeasy Blood & Tissue kit (Qiagen, UK) using the manufacturer's protocol, while total RNA extraction was performed using TriZol Reagent (Invitrogen) according to the manufacturer's instructions.

Synthesis of cDNA was performed on RNA from all 48 *T. vaginalis* strains using Random Examers and Superscript III Reverse Transcriptase in the presence of RNAse Inhibitor (Invitrogen) at 42 °C for 50 min.

Real-time PCR

The presence of TVVand *M. hominis* was assessed by quantitative PCR in cDNA and DNA samples, respectively (Jehee et al. 2017; Masha et al. 2017). Real-time PCR was performed using SYBR Select Master Mix (Applied Biosystems) according to the manufacturer's protocol (Invitrogen). Initial steps of 2 min at 50 °C and 10 min at 95 °C were followed by 40 cycles with denaturation at 95 °C for 15 s and annealing and extension that varied for each primer set. A dissociation curve was achieved by a gradual increase in temperature (0.5 °C). All real-time PCR assays were run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The resultant curves were analyzed by Biorad CFX Manager 3.1. Each experiment was performed in triplicate.

Metronidazole susceptibility assay

Metronidazole susceptibility was assessed in vitro for a selected group of 19 *T. vaginalis* strains both infected and not infected by the symbionts. Briefly, *T. vaginalis* cells in log growth phase were incubated in increasing levels of metronidazole (ranging from 0.2 to 100 μg/ml) in 96-well plates (1×10^5 cells/well) for 48 h in 5% CO_2. After 48 h of incubation, the plates

were microscopically observed and the minimum lethal concentration (MLC) was detected. All experiments were performed twice in triplicate for each isolated tested. Untreated cultures of each trichomonad strain were used as controls.

Immunofluorescence assay

Two representative *T. vaginalis* strains were used: SS-63, infected by all four species of *T. vaginalis* viruses and SS-22, naturally TVVs free. Cells were fixed with 4% paraformaldehyde for 1 h and incubated for 1 h with anti-dsRNA monoclonal antibody J2 (English & Scientific Consulting, HU) diluted 1:500. FITC-conjugated anti-mouse IgG antibody (Sigma, USA) was used as secondary antibody and 5 μg/ml 4′,6 diamidino-2-phenylindole (DAPI) for nucleic acid staining.
Samples were observed using an Olympus BX51 microscope and the images were acquired with Optronics MagnaFire CCD Camera.

Statistical analysis

Statistical analyses were performed using chi-square test (Microsoft Excel; Microsoft, USA). A $p < 0.05$ was considered significant.

Results

Trichomonas vaginalis virus detection in *T. vaginalis* isolates

In this work, 48 strains of Trichomonas vaginalis isolated in Italy were analyzed for the presence of the four species of *T. vaginalis* viruses (TVV1, TVV2, TVV3, and TVV4). For each isolate, total RNAwas extracted from exponential growing protozoa and reverse transcribed into cDNA, in order to assess the presence of TVVs by real-time PCR. Fifty percent of total *T. vaginalis* strains were found to be positive for at least one virus. The most prevalent virus was TVV2 (79.17% of positive *T. vaginalis* strains) followed by TVV1 (70.83%) and by TVV3 (54.17%). Only two *T. vaginalis* strains (8.33% of total positives) were infected by TVV4, always in association with other virus types. Eighteen *T. vaginalis* strains were infected by multiple species of virus (75% of positives), with one strain (SS-63) infected by all four viruses (see Table 1). In fact, five *T. vaginalis* strains were positive for only TVV2, one strain was exclusively associated with TVV1, while no strains positive for only for TVV3 or TVV4 were detected.

The most common association among viruses found in our group of *T. vaginalis* strains was between TVV1 and TVV2 (13 *T. vaginalis* strains out 24; 54.16%), followed by the association between TVV1 and TVV3 (11 *T. vaginalis* strains out of 24; 45.83%). Nine *T. vaginalis* strains have showed a co-infection with TVV2 and TVV3 (37.5%).

Pattern association between *M. hominis* and TVVs in *T. vaginalis* clinical isolates

Genomic DNA was extracted from all 48 *T. vaginalis* strains and analyzed by real-time PCR using primers specific for *M. hominis*. The presence of *M. hominis* was detected in 39 out of 48 *T. vaginalis* isolates (81.25%). Among them, 20 *T. vaginalis* strains tested positive also for at least one species of *T. vaginalis* virus. Statistical analyses found no correlation between *M. hominis* and one specific type of virus in *T. vaginalis* isolate analyzed. Interestingly, onlyfivetrichomonad strains were both Mycoplasma and TVV free (Table 1).

Association between TVVs symbiosis and metronidazole sensitivity

Experiments of in vitro metronidazole sensitivity were performed on 19 *T. vaginalis* representative of the different associations with symbionts. Results are described in Table 1. Most (95%) of *T. vaginalis* isolates were metronidazole sensitive, with MLC values ranging from 0.5 to 8.6 μg/ml. Statistical analysis showed no association between both TVV and *M. hominis* infection (P = 0.245 and 0.24, respectively) and MLC in *T. vaginalis*. Interestingly, the only *T. vaginalis* strain resistant to metronidazole, with MLC value of 50 μg/ml, is TVVs and *M. hominis* free.

Immunodetection of *T. vaginalis* virus

To assess the intracellular localization of TVVs, we set up an immunofluorescence test using the J2 monoclonal mouse antibody that allows the detection of various dsRNA viruses independently of their sequences (Lukacs 1994; Hyde et al. 2009; Zangger et al. 2013). Preliminary experiments conducted in Dot blot analysis demonstrated that the monoclonal antibody is able to detect all four TVVs in trichomonad cells (data not shown). Experiments were conducted on strain SS-22 (negative for all TVVs) and on strain SS-63 (infected by all four viruses subspecies), revealing the presence of numerous very small fluorescent spots uniformly distributed in the cytoplasm only in the TVV positive SS-63 strain (Fig. 1a). As expected, fluorescent spots were absent in SS-22 strain, which not contains TVV (Fig. 1b).

Discussion

In the last decades, the attention on *T. vaginalis* infection by dsRNA viruses has increased, since they are considered able to influence trichomonad virulence and pathogenesis (Wendel et al. 2002). Several studies carried out in different part of the world have reported the existence of four types of viruses that can coexist in different combinations in the same trichomonad cell (Benchimol et al. 2002; Fraga et al. 2012; Goodman et al. 2011; Rivera et al. 2017).

In the current study, we investigated, for the first time, the presence of TVVs in a group of *T. vaginalis* strains isolated in Italy. We analyzed the possible association with the presence of *M. hominis* and with the sensitivity to metronidazole.

Fifty percent of our isolates tested positive for at least one TVV species in real-time PCR. The TVV prevalence in Italian *T. vaginalis* is similar to that observed in most countries. Only in a few studies, carried out in Iran, India, Egypt, South Korea, and Philippines, the prevalence rate of TVV infection was very low (14–20%), probably due to different technical approaches (Fichorova et al. 2017; Graves et al. 2019a).

Our study showed that TVV2 is the most prevalent virus associated with Italian trichomonad strains, followed by TVV1 and TVV3 with TVV4. These results differ from those obtained by Graves et al. (2019b) in the USA, where the most prevalent virus is TVV1, followed by TVV2, TVV3, and TVV4.

Interestingly, two Italian strains tested positive for TVV4, showing for the first time the presence of this virus species in Europe. *T. vaginalis* cells can be infected by more than one TVVs species simultaneously. The ability of different viral species to coexist in a single host is common among Totivirus and Victovirus, being these viruses largely non-cytopathic and able to well-adapt in the host cell environment (Goodman et al.2011). Anyway, Fichorova et al. (2017) reported that the majority of *T. vaginalis* strains tested so far in different countries were infected with a single TVV species. Interestingly, we found that the 75% of the Italian trichomonad strains tested in this work were infected by more than one TVV species. It has been suggested (Rivera et al. 2017) that the occurrence of multiple TVVs may be due to an infection by more than one *T. vaginalis* strain in the same patient, rather than to the concurrent infection of different TV viruses in a single protozoan cell. We tend to exclude a multiple infection by different *T. vaginalis* strains in our patient group, since the prevalence of trichomoniasis in Italy is very low (Diaz et al. 2010; Camporiondo et al. 2016).

The presence of multiple TVV types in a single *T. vaginalis* cell may play important roles in upregulating inflammatory reaction and in development of trichomoniasis. Several studies reported an increased severity of symptoms of trichomoniasis in patients with TVVs positive protozoa. The presence of viruses in protozoan cells may also influence their growth: Khanaliha et al. (2017) have reported that *T. vaginalis* isolates infected by TVVs showed a high growth rate and the ability

to reach a higher density compared with *T. vaginalis* isolates lacking viral infections. On the other hand, Rivera et al. (2017) observed that the presence of TVV rendered *T. vaginalis* cells harder to maintain in culture with the exception of the only trichomonad strain harboring simultaneously the four TVVs. Among our *T. vaginalis* isolates,we have observed that generally, there were no differences among protozoa infected and uninfected by viruses in terms growth rate and density. Interestingly, differently from results obtained by Rivera and colleagues, we noticed that our *T. vaginalis* isolate infected by all four TVVs was very difficult to cultivate in vitro (data not shown).

We investigated on the intracellular localization of TVVs in *T. vaginalis* cells using specific anti dsRNA antibody.

TVVs are uniformly distributed throughout most of the cytoplasm. Our results shed light for the first time on the location of TVVs inside protozoan cells. The intracellular distribution of viral dsRNA in *T. vaginalis* is similar to the one observed in Leishmania species for Leishmaniavirus by Zangger et al. (2013). Previous studies have highlighted the capability of *T. vaginalis* to establish a symbiosis with *Mycoplasma hominis*. In this work, we analyzed by real-time PCR the possible correlation between the presence of *M. hominis* and the different TVV species. We found that 81% of trichomonad strains are in stable endosymbiotic relationship with *M. hominis*, confirming the results previously obtained in the same geographical area (Rappelli et al. 1998). We demonstrated that 51.28% of *T. vaginalis* isolates harboring *M. hominis* were infected by at least one type of TVVs. A similar percentage was observed among the protozoa not infected by *M. hominis* (44.4%). Similarly, no statistical correlation was demonstrated between the presence of *M. hominis* and a single species of virus. In our study, only 10.41% of trichomonad strains are free from both Mycoplasma hominis and TVVs. Taken together, these results confirmed the ability of *T. vaginalis* to adapt to symbiosis with different microorganisms that may be emerging key players influencing the outcome of parasitic disease.

The possible correlation between metronidazole resistance and the presence of TVVs and *M. hominis* associated with *T. vaginalis* was evaluated. The impact of symbionts on protozoan resistance to antibiotics is an issue still debated. Some studies suggested a positive correlation between infection by viruses (Snipes et al. 2000; Hampl et al. 2001) or by *M. hominis* and resistance to metronidazole, but these results are in contrast with those reported by other authors (Dessì et al. 2019). In this work, we reported the lack of relationship between the presence of TVVs and metronidazole resistance, with MLC varying among the different strains independently from the presence of any symbiont. The overall sensitivity to metronidazole is of 95.3%. Interestingly, in our study, the only *T. vaginalis* strain resistant to metronidazole is TVVand *M. hominis*-free, consistently with the observations reported by da Luz Becker and colleagues (da Luz et al. 2015) on a group of trichomonad isolates from Brazil. These findings suggest the importance of further studies to shed light on the relationship between drug resistance in *T. vaginalis* and the presence of the different symbionts.

This study is the first report on the prevalence of TVVs in Italy and it is the second one in Europe. Our data showed the high prevalence of trichomonad strains infected with both different types of TVVs and *M. hominis*, displaying the capability of protozoan to establish endosymbiotic relationship with several microorganisms simultaneously.

Moreover, the interaction among TVVs, *M. hominis*, and *T. vaginalis* need to be may modify host response and the pathogenesis of protozoan, suggesting a new field of research to explore.

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Table 1*: Trichomonas vaginalis* isolates infection by TVV species and *Mycoplasma hominis*, and 271 Minimal Lethal concentration values (MLC) for metronidazole

T. vaginalis	TVV1	TVV2	TVV3	TVV4	*M. hominis*	MLC (µg/ml)
SS-01	+	+	+	-	+	3,13
SS-02	+	+	+	-	+	N.D.
SS-03	-	-	-	-	+	N.D.
SS-06	-	-	-	-	+	N.D.
SS-09	+	+	+	-	+	N.D.
SS-10	-	+	+	-	+	0,5
SS-11	+	-	+	-	+	6,25
SS-13	+	+	-	-	+	2,1
SS-14	-	-	-	-	+	1,1

SS-15	+	+	+	-	+	2,1
SS-16	+	+	+	-	+	N.D.
SS-17	-	+	-	-	+	8,6
SS-18	-	+	-	-	+	N.D.
SS-20	-	-	-	-	+	N.D.
SS-22	-	-	-	-	-	50
SS-25	+	+	-	-	+	N.D.
SS-26	-	-	-	-	+	2,1
SS-31	-	-	-	-	+	N.D.
SS-32	-	-	-	-	+	1,56
SS-35	+	-	+	-	+	1,1
SS-36	-	-	-	-	+	6,25
SS-41	-	-	-	-	+	N.D.
SS-42	+	+	-	-	+	N.D.
SS-45	-	-	-	-	+	1,1
SS-47	-	-	-	-	+	N.D.
SS-48	+	-	+	-	-	1,1
SS-49	-	-	-	-	+	N.D.
SS-60	+	+	-	-	+	3,13
SS-62	-	-	+	+	-	N.D.
SS-63	+	+	+	+	-	N.D.
SS-78	+	+	-	-	+	N.D.
SS-77	-	+	-	-	+	8.6
SS-81	-	-	-	-	-	N.D.
SS-84	-	-	-	-	+	N.D.
SS-85	-	-	-	-	-	N.D.
SS-86	-	-	-	-	-	N.D.
SS-87	-	-	-	-	+	N.D.
SS-88	-	+	-	-	+	1,1
SS-89	+	+	+	-	-	N.D.
SS-90	-	-	-	-	+	N.D.
SS-U-1	-	-	-	-	-	1,56
NU-01	-	-	-	-	+	N.D.
NU-02	-	-	-	-	+	N.D.
NU-03	-	-	-	-	+	N.D.
TO-1	-	-	-	-	+	N.D.
TO-3	+	-	-	-	+	2,1
TO-4	+	+	+	-	+	N.D.
MO-4	-	+	-	-	+	N.D.

Fig 1: Immunolocalization of TVVs in *Trichomonas vaginalis.*

Green: anti-dsRNA J2 monoclonal antibody. Blue: DAPI.

a*: T. vaginalis* strain SS-63 (TVV1, TVV2, TVV3 and TVV4 positive). b: *T. vaginalis* strain SS-22 280 (TVV negative)