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Trichomonas vaginalis and *Mycoplasma hominis*: new tales of two old friends

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Review Article

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Abstract

Trichomonas vaginalis is an anaerobic protist, responsible for the most prevalent non-viral sexually transmitted infection in humans. One of the most intriguing aspects of *T. vaginalis* pathobiology is the complex relationship with intracellular microbial symbionts: a group of dsRNA viruses belonging to family of *Totiviridae* (*T. vaginalis* virus), and eubacteria belonging to the *Mycoplasma* genus, in particular *Mycoplasma hominis*. Both microorganisms seem to strongly influence the lifestyle of *T. vaginalis*, suggesting a role of the symbiosis in the high variability of clinical presentation and sequelae during trichomoniasis. In the last few years many aspects of this unique symbiotic relationship have been investigated: *M. hominis* resides and replicates in the protozoan cell, and *T. vaginalis* is able to pass the bacterial infection to both mycoplasma-free protozoan isolates and human epithelial cells; *M. hominis* synergistically upregulates the proinflammatory response of human monocytes to *T. vaginalis*. Furthermore, the influence of *M. hominis* over *T. vaginalis* metabolism and physiology has been characterized. The identification of a novel species belonging to the class of *Mollicutes* (Candidatus *Mycoplasma girerdii*) exclusively associated to *T. vaginalis* opens new perspectives in the research of the complex series of events taking place in the multifaceted world of the vaginal microbiota, both under normal and pathological conditions.

Introduction

Protists and bacteria have co-evolved for the past ~1.5 billion years. Often protozoans predate bacteria but, in some cases, these bacteria are able to resist intracellular killing establishing symbiosis with these single-cell eukaryotes. The endosymbiotic acquisition of a variety of plastids and mitochondrion-related organelles in protists are clear evidence that endosymbiosis provides innovation and significant benefits to the microorganisms involved in these relationships (Nowack and Melkonian, 2010).

Differently from environmental protists, eukaryotic human pathogens are often believed to be free of parasitic bacteria. Several protozoa colonize sterile niches in humans (e.g. blood, central nervous system), but other species share the same environment with billions of microorganisms (e.g. gut and vagina) and show a marked inclination to phagocytize bacteria.

The interest of clinicians for microbial endosymbiosis has been triggered only in 1976, after an outbreak of pneumonia involving a significant number of attendees at a convention of the American Legion in Philadelphia. Among more than 2000 legionnaires, 182 contracted a severe pneumonia, with 29 deaths. The aetiological agent of pneumonia was a 'new' bacterium, *Legionella pneumophila*. *L. pneumophila* is able to grow and spread in human-made complex water systems (such as air-conditioning, SPAs, etc.). Interestingly, *L. pneumophila* can be isolated from cooling towers: these bacteria are protected from extreme temperatures when hosted as endosymbionts of *Acanthamoeba*, a free-living amoeba whose cysts are able to resist to environmental stresses such as extreme pH, high temperatures and chlorine. The cystic form of *Acanthamoeba* not only protects the endosymbiotic *Legionella* and but also represents the main environmental reservoir for this bacterium (Jules and Buchrieser, 2007).

Trichomonas vaginalis is a human pathogenic protozoon, responsible for the most common non-viral sexually transmitted infection worldwide, with 170 million new cases each year (World Health Organization, 2012). The clinical presentation of trichomoniasis in women may vary from asymptomatic to severe vaginitis, while the infection is mainly asymptomatic in men. Trichomoniasis is associated with a number of pregnancy and postpartum complications, such as preterm birth, premature membrane rupture and low birth-weight (Petrin *et al.*, 1998; Fichorova, 2009; Edwards *et al.*, 2014; Hirt and Sherrard, 2015). Furthermore, *T. vaginalis* infection has been associated with an increased risk of invasive cervical cancer (Yap *et al.*, 1995), prostate cancer (Sutcliffe *et al.*, 2006) and of human immunodeficiency virus (HIV) acquisition and shedding (McClelland *et al.*, 2007). The latest concepts on the mechanisms by which *T. vaginalis* exerts its pathogenic effects focus on adhesion to and damage of host epithelial cells, subversion of host immunity and induction of inflammation and possibly disruption of the vaginal microbiota (Mercer and Johnson, 2018). The standard treatment for trichomoniasis is the oral administration of metronidazole, an antimicrobial drug which has been shown to be highly effective in *T. vaginalis* infection management, although the global

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circulation of resistant protozoan strains is increasingly becoming a matter of concern (Kissinger, 2015).

In 1998, our group demonstrated the presence of viable mycoplasmas in *T. vaginalis* clinical isolates. The bacteria were identified as *Mycoplasma hominis* by polymerase chain reaction (PCR) and were reisolated in specific growth medium (Rappelli *et al.*, 1998).

Since its first description, several groups demonstrated by PCR the presence of *M. hominis* in trichomonad isolates of different geographic origin, with infection rates ranging from 5% to over 89% (Hampl *et al.*, 2001; Van Der Schee *et al.*, 2001; Xiao *et al.*, 2006; Butler *et al.*, 2010; Diaz *et al.*, 2010; Fraga *et al.*, 2012; da Luz Becker *et al.*, 2015). For a review see Fichorova *et al.* (2017). The intracytoplasmic location of *M. hominis* in *T. vaginalis* cells has been demonstrated by gentamicin protection assays and by confocal and electron microscopy (Dessì *et al.*, 2005; Vancini and Benchimol, 2008). *Mycoplasma hominis* can be transmitted from *M. hominis*-infected *T. vaginalis* to *M. hominis*-free protozoa and to human cell lines *in vitro* (Rappelli *et al.*, 2001).

Mycoplasma hominis is an obligate parasite of the human urogenital tract where it can be responsible for bacterial vaginosis, among many other clinical manifestations and signs.

The symbiosis between *T. vaginalis* and *M. hominis* represents the first reported and unique case of an endosymbiotic association between two obligated human parasites, able to produce infections in the same anatomical site and causing independent diseases in humans. Interestingly, both pathogens are associated with pregnancy and post-partum complications, including premature rupture of fetal membranes, preterm delivery and low-birth-weight infants (Pararas *et al.*, 2006; Wen *et al.*, 2014).

Mycoplasma hominis shows very peculiar features and has one of the smallest genomes among self-replicating organisms: it lacks a cell wall, its membrane contains sterols and notably hydrolyses arginine as its major energy source (Razin *et al.*, 1998; Pereyre *et al.*, 2009). Pathogenicity mechanisms are thought to involve invasion of and replication within human cells, resistance to intracellular killing and multiplying in the cytoplasm (Hopfe *et al.*, 2013; Henrich *et al.*, 2017), as well as host inflammatory response. *Mycoplasma hominis* lipoproteins can stimulate interleukin (IL)-23 production by dendritic cells and macrophages (Starlets *et al.*, 2006; Fiori *et al.*, 2013; Goret *et al.*, 2017), thus contributing to generate a significant proinflammatory response during infection.

The intracellular location is a privileged niche, where bacteria are 'invisible' to host recognition mechanisms of the immune system, and are potentially protected from the cytocidal effect of many antibiotics. In this case, *T. vaginalis* plays a role as a Trojan horse, with a direct benefit for its bacterial symbiont. Similarly, as described later in this review, it is likely that trichomonads may gain considerable advantages from this relationship, leading to a stable maintenance of this microbial relationship.

In the last few years, we focused our attention on some fundamental aspects of the endosymbiotic relationship between *T. vaginalis* and *M. hominis*: (i) the modulation of the host inflammatory response, (ii) the influence on some peculiar biochemical pathways (particularly on arginine metabolism) and the role of *M. hominis* (iii) during pregnancy and (iv) in tumourigenesis. These topics will be further detailed and discussed in this review.

Impact of the *T. vaginalis/M. hominis* symbiosis over immunopathogenesis of trichomoniasis

The current model of *T. vaginalis* pathogenesis involves two main aspects, each contributing to the host tissue damage: on the one hand, the cytopathic effect exerted by the direct action of trichomonad cells through adhesion and secretion of toxic molecules; on the other hand, the strong inflammatory response often observed during trichomoniasis also contributing to pathology (Fiori *et al.*, 1999; Fichorova, 2009; Ryan *et al.*, 2011; Figueroa-Angulo *et al.*, 2012).

Though this symbiosis was first described about 20 years ago, only a few studies dealing with the influence of this relationship over *T. vaginalis*-mediated cytotoxicity have been described in the literature.

In detail, Vancini and colleagues set up an *in vitro* model of interaction between *T. vaginalis* and primary vaginal epithelial cells (VECs). *Mycoplasma hominis*-infected *T. vaginalis* were shown to induce more cellular damage to VECs in culture as compared with uninfected control trichomonads.

The same effect was not observed when the microorganisms were used to infect the canine kidney cell line MDCK, suggesting how this phenomenon might be tissue- and species-specific. Quite consistently with these observations, Vancini and colleagues observed in the same article that *M. hominis*-harbouring *T. vaginalis* also showed an increased amoeboid transformation rate and enhanced ability to phagocytize *Saccharomyces cerevisiae* yeast cells (Vancini *et al.*, 2008).

Though the mechanisms underlying these observations were not investigated, the authors suggested that the presence of *M. hominis* symbiotically associated with *T. vaginalis* might account for increased parasite virulence *in vitro*.

While in the study by Vancini and coworkers the comparison between *mycoplasma*-infected and -uninfected *T. vaginalis* cells was made possible thanks to the experimental infection of a naturally *mycoplasma*-free *T. vaginalis* isolate with a *M. hominis* pure culture, Mercer and colleagues set up an experimental system using an alternative and rigorous approach (Mercer *et al.*, 2016). The authors produced an isogenic *mycoplasma*-free *T. vaginalis* strain by means of an antibiotic treatment of a naturally *mycoplasma*-infected parasite isolate, and they tested the influence of *M. hominis* over *T. vaginalis* ability to kill human primary B-cells, T-cells and monocytes. Both experimental approaches to obtain two *T. vaginalis* isogenic strains, one mycoplasma-free and one mycoplasma-infected, allow us to rule out the extreme variability of the virulence traits among trichomonad strains, likely due to their natural genetic diversity. The authors characterized the trichomonad-mediated cell death, with a preference for B-cells over T-cells, while the lysis of monocytes was far less efficient. Interestingly, and partially in contrast with the observations of the work from Vancini *et al.*, the presence of *M. hominis* showed no effect on *T. vaginalis* cytolytic activity, but had a marked effect on the inflammatory response of monocytic cells. *Mycoplasma hominis* induced a qualitative and quantitative modification of the proinflammatory cytokines secreted by monocytes in response to *T. vaginalis* infection. Indeed, *M. hominis* is a well-known activator of host immunity, especially of Toll-like receptor-2-mediated inflammatory response (Peltier *et al.*, 2005; Truchetet *et al.*, 2011; Hasebe *et al.*, 2014). One speculative observation of the work by Mercer and collaborators could point out how the strong antibiotic regime *per se* may have influenced on the gene expression of virulence genes in *T. vaginalis* possibly by epigenetic changes.

Inflammation appears to play a key role in *T. vaginalis* pathogenesis: a heavy leucocyte infiltration is frequently observed during trichomoniasis, accompanied by significant levels of secreted proinflammatory cytokines (Shaio *et al.*, 1995; Simhan *et al.*, 2007). Indeed, *T. vaginalis*-mediated inflammation has been proposed to explain the increased risk of HIV acquisition, and of prostate and cervical cancer, associated with trichomoniasis (Yap *et al.*, 1995; Sutcliffe *et al.*, 2006; McClelland *et al.*, 2007). The local inflammation induced by *T. vaginalis* attracts at the

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site of infection many immune cells permissive to HIV infection such as dendritic cells, monocytes and $CD4^+$ lymphocytes (Thurman and Doncel, 2011). In a similarly speculative fashion, it has been proposed that the subclinical inflammatory status of asymptomatic *T. vaginalis* infections might contribute to prostate and cervical cancer onset and progression (Sutcliffe *et al.*, 2012).

Given the paramount importance of inflammatory phenomena in *T. vaginalis* immunopathogenesis, our research group investigated the influence of the mycoplasmal symbiont over the host innate immunity.

We showed how *M. hominis* is able to synergistically upregulate the secretion of proinflammatory cytokines (IL-8, IL-1β, tumour necrosis factor-α) by the human monocytic cell line THP-1 in response to *T. vaginalis* stimulation. Interestingly IL-23, a Th17-polarizing cytokine, is secreted only after stimulation with mycoplasma-infected trichomonad cells. The use of a THP-1 reporter cell line allowed us to show how the activity of the transcription factor nuclear factor (NF)-κB is similarly upregulated by the presence of the bacterial symbiont *M. hominis*. This suggests the involvement of intracellular pathways leading to an increased activation and nuclear translocation of NF-κB (Fiori *et al.*, 2013).

The presence of *M. hominis* in *T. vaginalis* isolates might play a key role in inflammation during trichomoniasis, thus potentially affecting the severity of the disease. The upregulation of the macrophage proinflammatory response might also affect several clinical conditions associated with *T. vaginalis* infection, such as the increased risk of acquiring cervical cancer, prostate cancer or HIV, which are thought to be affected by the host inflammatory response to this parasite.

Altogether these data suggest that the symbiotic association between *T. vaginalis* and *M. hominis* is potentially involved in the modulation of host cell cytotoxicity and of immune-mediated pathogenesis.

Mycoplasma hominis impact on metabolic pathways and physiopathology of *T. vaginalis*

In the last few years, several studies have highlighted the mutualistic nature of this microbial relationship, supporting a model by which both microorganisms benefit from this association. Particular interest has been raised after revealing that *M. hominis* provides metabolic benefits to *T. vaginalis*. In detail, Morada and colleagues have studied a shared biochemical pathway, the arginine dihydrolase (ADH) pathway, which leads to conversion of arginine to ornithine and ammonia through the enzymes arginine deiminase (ADI), catabolic ornithine carbamoyltransferase (OCT) and carbamate kinase (CK), with final production of adenosine triphosphate (ATP) by substrate phosphorylation (Yarlett *et al.*, 1996).

Trichomonas vaginalis rapidly depletes arginine from growth medium during cultivation, obtaining up to 10% of energy requirements through ADH pathways under anaerobic conditions (Linstead and Cranshaw, 1983; Zuo *et al.*, 1995). Moreover, this pathway becomes particularly important for the survival of the protozoan under glucose restriction, where both OCT and CK are upregulated in the log growth phase (Huang *et al.*, 2014). In *M. hominis*, ADH pathway is the major energy pathway (Pereyre *et al.*, 2009). *T. vaginalis* symbiotically associated with *M. hominis*, potentially equipped with two ADH pathways of distinct evolutionary origins, competing for the same substrate (arginine), exhibits increased arginine consumption, concomitant with an increase in ornithine and putrescine production, 16- fold and 3-fold higher respectively, as compared with mycoplasma-free trichomonads (Morada *et al.*, 2010). *Mycoplasma hominis* could benefit from the scavenging of a constant supply of putrescine, which is not capable of synthesizing, represented by an increased putrescine production by *T. vaginalis* ornithine decarboxylase when associated with *M. hominis* (Shah and Swiatlo, 2008). Interestingly, addition of free arginine to culture medium is characterized by an increase in the amount of ATP/cell in *T. vaginalis–M. hominis* consortium (Margarita *et al.*, 2016). The overall increase of ATP in protozoan–bacterium consortium is likely due to mycoplasma ADH pathways, since the expression of all *T. vaginalis* ADI genes is not upregulated by the bacterial symbionts (Morada *et al.*, 2010). Despite the intuitive view that both microorganisms may compete for the increased ATP availability, several experimental observations may lead to an integrated view supporting the idea that the metabolic interaction between *M. hominis* and *T. vaginalis* may promote a mutual benefit. We observed a boost in *T. vaginalis–M. hominis* growth rate (~20%) and a higher cell density (~40%) in the log phase as compared with protozoa alone, supporting the hypothesis that *M. hominis* may enhance the growth rate of *T. vaginalis* by increasing intracellular ATP, since intracellular ATP concentration is linked with the growth rate of several parasites (Miyahira, 1991).

Moreover, we recently showed that the presence of *M. hominis* symbiotically associated to *T. vaginalis* contributes to subtraction of arginine from growth medium in a co-culture system with human macrophages. This leads to the inhibition of nitric oxide (NO) production potentially affecting the NO-mediated killing abilities of phagocytes (Margarita *et al.*, 2016). The competition for the uptake of free arginine from the environment between *T. vaginalis* in symbiosis with *M. hominis* and macrophages may be carried out by ADI, since several studies suggest a central role of this enzyme in microbial virulence (Yarlett *et al.*, 1996; Fulde *et al.*, 2011). Moreover, regulation of arginine availability is a common strategy used by several pathogens including *M. hominis* to evade antimicrobial NO production by human macrophages (Noh *et al.*, 2002; Bronte and Zanovello, 2005).

In addition, mycoplasma-infected *T. vaginalis* showed an increased haemolytic activity *in vitro* (Margarita *et al.*, 2016), suggesting a further contribution of the symbiosis to *Trichomonas* pathobiology.

The possible correlation between metronidazole resistance and the presence of *M. hominis* associated with *T. vaginalis* has received much attention, and this issue is still debated. Several studies carried out on *T. vaginalis* strains isolated in different geographical areas suggest a lack of a relationship between metronidazole susceptibility and the presence of *M. hominis* (Fichorova *et al.*, 2017). Other studies, by contrast, report a positive association between resistance to metronidazole in *T. vaginalis* and the presence of the bacterial symbiont. A recent study has evaluated the impact of *M. hominis* infection on the RNA expression levels of three genes associated with metronidazole resistance in *T. vaginalis*. Results showed a downregulation of metronidazole susceptibility associated genes in all *T. vaginalis* strains tested in presence of *M. hominis* (Fürnkranz *et al.*, 2018), albeit with a limited statistical significance. Altogether, the studies present in the literature are scarce and contradictory, and all show the lack of a systematic approach to analyse a significant number of protozoan isolates. Further studies are needed to clearly show a direct correlation between the presence of *M. hominis* and *T. vaginalis* drug resistance.

The role of *T. vaginalis*/*M. hominis* symbiosis on adverse pregnancy outcomes

Coincidentally, *T. vaginalis* and *M. hominis* are both associated with increased adverse pregnancy outcomes. *T. vaginalis* is a non-invasive pathogen unable to invade mucosal surfaces and fetal membranes during pregnancy, limiting its colonization to the

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vaginal tract. *Mycoplasma hominis* in contrast can be isolated from infected placental membranes and amniotic fluid, with a potential direct effect of virulence mechanisms in this microenvironment. A partial explanation for a role of *T. vaginalis* in adverse pregnancy outcomes is given by the host immune response induced by infection, characterized by a local inflammation: the subsequent production of cytotoxic cytokines can indirectly lead to severe pregnancy complications.

Recently, intra-amniotic infections with high risk of preterm delivery has been associated with *M. hominis* carrying the gene *goiC* (Allen-Daniels *et al*., 2015). We have recently demonstrated that 58% of *M. hominis* isolated from *T. vaginalis* carry this gene, suggesting an additional potential risk factor for adverse pregnancy outcomes during trichomoniasis (Thi Trung Thu *et al*., 2018).

The intracellular localization of mycoplasmas in *T. vaginalis* cells can explain the paradoxical results described by some authors, reporting the failure of metronidazole treatment of subclinical trichomoniasis to prevent adverse pregnancy outcomes. In fact, selective treatment of trichomoniasis with metronidazole could induce a massive release of *M. hominis* from dead parasites leading to bacterial invasion of placental membranes and amniotic fluid (Klebanoff *et al*., 2001; Thi Trung Thu *et al*., 2018).

Similarly, Fichorova *et al*. demonstrated that metronidazole treatment induces a massive release of *T. vaginalis* virus (TVV). The virus particles and the free dsRNA strongly activate host innate immune-response (Fichorova *et al*., 2012).

The role of *T. vaginalis*/*M. hominis* symbiosis in tumourigenesis

Trichomoniasis is associated with malignant transformation and cancer maintenance and may be considered a potentially carcinogenic agent. Indeed, *T. vaginalis* infections are associated with cervical and prostate cancer (Sutcliffe, 2006; Stark *et al*., 2009; Tao *et al*., 2014). Recently, a direct molecular mechanism in prostate tumour transformation and progression based on the secretion of a protozoan homologue of the human macrophage migration inhibitory factor (MIF) has been proposed. MIF is a pleiotropic proinflammatory cytokine able to promote oncogenesis and is a key molecule in prostate cancer (Twu *et al*., 2014).

The subclinical nature of human infection by *M. hominis* suggests that it could generate chronic inflammation with procancerous effects. Numerous studies have shown that chronic mycoplasma infection *in vitro* can result in chromosomal aberration, genetic instability, DNA fragmentation due to bacterial nucleases and malignant transformation (Namiki *et al*., 2009; Barykova *et al*., 2011; Gagnaire *et al*., 2017). Interestingly, a computational prediction study has identified a number of *M. hominis* proteins putatively involved in prostate cancer (Zakariah *et al*., 2018). It could be hypothesized that *M. hominis*, transported and protected by *T. vaginalis*, is released from the protozoan cells after metronidazole treatment and immune-mediated cell death (Mercer *et al*., 2018), and that the subsequent invasion of cervical and prostate cells by *M. hominis* could contribute to malignant transformation or tumour progression.

Conclusions and perspectives

Pathogenesis of trichomoniasis has been historically investigated as a balance between parasite virulence and host immune response. However, *T. vaginalis* displays a complex trophic level of interactions with other microorganisms including endosymbionts (mycoplasma and TVV) and commensal bacteria of the urogenital tract. *Trichomonas vaginalis*, endosymbionts and associated microbiota make this unique poly-microbial entity which apparently modifies host response and parasite virulence. Therefore, these microbial interactions might be very influential to this disease and this is a very exciting field of research (Bär *et al*., 2015; Mercer and Johnson, 2018).

Since the majority of *T. vaginalis* clinical isolates is stably infected by *M. hominis*, the acquisition of trichomonad infection implies the simultaneous acquisition of *M. hominis*. The complex poly-microbial interactions involving *T. vaginalis*, its endosymbionts (mycoplasmas and TVV) and the vaginal microbiota, affect the general mucosal integrity and might influence the host inflammatory response, thus increasing HIV susceptibility and altogether affecting reproductive health. The *T. vaginalis*/*Mycoplasma* consortium is an example of a pathogroup: a combination of low virulent microorganisms that together are able to potentially produce aggressive diseases. *Mycoplasma hominis* can influence the pathogenicity of *T. vaginalis*, suggesting a role of *T. vaginalis*/*M. hominis* symbiosis in the high degree of variability of signs and symptoms observed during trichomoniasis.

Recently, in a metagenomic study of the human vaginal microbiome, a new *Mycoplasma* species has been found exclusively in women positive for *T. vaginalis*. The bacterium, named Candidatus *Mycoplasma girerdii*, is not culturable *in vitro*, showing why it was not identified before in the vaginal environment (Fettweis *et al*., 2014). Candidatus *Mycoplasma girerdii* has a small genome of 619 kb (with 28.6% GC content) with very limited metabolic pathways. The presence of Ca. *M. girerdii* has been also demonstrated in the oral cavity of a preterm infant (Costello *et al*., 2017). Notably, Ca. *M. girerdii* genome sequencing highlighted the presence of putative virulence genes such as haemolysin, endopeptidase and a collagenase. The role of this new *Mycoplasma* species in modulating *T. vaginalis* pathobiology is unknown and further studies may identify its potential role in all the aspects discussed in this review article.

Based on the clues and hypotheses raised in this review, future experimental research could tackle the various aspects of this symbiotic relationship which remain unknown or debatable. Importantly, the ability of these symbionts to cooperate for immune evasion strategies is mostly unknown. This knowledge will help understating the basis of chronic subclinical *T. vaginalis*/*M. hominis* infections.

Specifically, further studies should investigate the potential role of *M. hominis* in modulating the response of other innate immune cells to *T. vaginalis*, such as dendritic cells and neutrophils.

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