UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Trichomonas vaginalis: citoaderência às células epiteliais vaginais *in vitro* e adesão às superfícies abióticas

ODELTA DOS SANTOS ALLENDE

PORTO ALEGRE, 2016

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Tese apresentada por **Odelta dos Santos Allende** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas.

Orientadora: Profª. Dr. Tiana Tasca
Coorientador: Prof. Dr. Alexandre José Macedo

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RESUMO

Introdução: *Trichomonas vaginalis* é um protozoário extracelular, agente etiológico da tricomonose, a mais prevalente doença sexualmente transmissível (DST) não viral no mundo. A citoaderência tem sido um dos mecanismos de patogenicidade mais estudados, porém, pouco se avaliou sobre a adesão do *T. vaginalis* a superfícies abióticas e, até o presente momento, não foi determinado se o *T. vaginalis* adere a dispositivos intrauterinos (DIU) e ao anel vaginal. **Objetivos:** (i) avaliar a capacidade de adesão do *T. vaginalis* às diferentes superfícies abióticas e estudar os mecanismos envolvidos nesse processo; (ii) comparar os mecanismos envolvidos na citoaderência com os de adesão a superfícies abióticas. No decorrer do estudo, ficou evidenciada a necessidade de inclusão de dois objetivos: (a) uma revisão sistemática da literatura científica para determinar as estimativas de prevalência e incidência da tricomonose no mundo; (b) determinar a estabilidade de genes candidatos a genes de referência para a utilização como *housekeeping* genes nos ensaios de expressão gênica. **Métodos:** Foi realizada uma revisão sistemática, seguindo as normas do *PRISMA guidelines*, para avaliar números atuais de prevalência e incidência da tricomonose no mundo. Com relação à estabilidade de genes candidatos a genes de referência, nove tiveram seus níveis de expressão constitutiva avaliados quando cultivados sob limitação e suplementação de nutrientes. Trinta e dois isolados de *T. vaginalis* foram avaliados quanto à capacidade de adesão ao plástico, pelo método de cristal violeta em placas de poliestireno. Na sequência, foram selecionados, aleatoriamente, dois isolados que apresentaram forte adesão ao plástico para serem utilizados como modelo de estudo dos mecanismos de citoaderência às células epiteliais vaginais (CEVs) e adesão às superfícies abióticas (DIU e anel vaginal). Os isolados de *T. vaginalis* TV-LACM6 e TV-LACM14 foram tratados com meta-periodato, colchicina, tripsina, citochalasina e metronidazol e reavaliados quanto à capacidade de adesão e citoaderência. Foram determinados os níveis de expressão gênica de quatro adesinas (AP33, AP51, AP65 e AP120) em trofozoítos aderidos em placas de poliestireno e às CEVs. Os isolados ATCC 30236, TV-LACM6 e TV-LACM14 foram usados na determinação da adesão ao DIU e ao anel vaginal e a adesão a esses dispositivos foi confirmada por microscopia confocal. **Resultados:** A revisão sistemática da literatura demonstrou que são raros os estudos

quanto à prevalência e incidência da tricomonose no mundo. Reafirma-se, portanto, a posição da tricomonose como uma doença parasitária negligenciada. Dentre os nove candidatos a genes de referência (*actina, F-actina* (*β* e *α*), *tubulina* (*α***,** *β* e *γ*), *gliceraldeído-3-fosfato desidrogenase, fator de elongamento* e *DNA topoisomerase II*), os genes *α-tubulina*, *actina* e *DNATopII* foram os mais estáveis, nas condições de limitação de nutrientes. Dos 32 isolados avaliados, 59 (35%) aderiram ao plástico. Após os tratamentos químicos observou-se que os isolados TV-LACM6 e TV-LACM14 reduziram os níveis de adesão tanto às CEVs quanto ao plástico, demonstrando que o *T. vaginalis* realiza a patogênese através de adesão e citoaderência por múltiplos mecanismos, os quais envolvem a participação do lipofosfoglicano e proteínas de membrana. Na análise da expressão gênica constatou-se que nos trofozoítos aderidos às CEVs houve aumento na expressão das quatro adesinas (AP33, AP51, AP65 e AP120). Por outro lado, nos trofozoítos aderidos ao plástico não existiu aumento nos níveis de expressão desses genes. Os isolados TV-LACM6 e TV-LACM14 aderiram ao DIU e ao anel vaginal, já o isolado ATCC 30236 apresentou praticamente nula adesão a tais dispositivos. **Conclusões:** Considerando a revisão sistemática da literatura constatou-se que existem poucos estudos, com qualidade metodológica, quanto à prevalência e incidência da tricomonose, assim pouco se sabe sobre a realidade epidemiológica dessa doença. A validação de genes de referência originou o primeiro relato deste tipo de estudo em *T. vaginalis,* no qual demonstramos que os genes mais estáveis foram *α-tubulina, actina e DNATopII*; por outro lado, os mais instáveis foram *GAPDH e β-tubulina.* Em relação ao estudo da adesão de *T. vaginalis* a superfícies abióticas concluímos que os trofozoítos de *T. vaginalis* aderem no DIU e ao anel vaginal e é inédita a descrição desse fenômeno. Ao analisarmos os mecanismos envolvidos na adesão e citoaderência concluímos que o LPG tem um papel importante em ambos os processos, por outro lado, as adesinas de superfície parecem não estar envolvidas na adesão a superfícies abióticas (plástico). Finalmente, nossos resultados reafirmam a importância do *T. vaginalis* como um importante problema para mulheres em idade reprodutiva, pois, a adesão aos dispositivos contraceptivos poderá facilitar a infecção. Além disso, nossos resultados indicaram uma possível diferença entre o processo de citoaderência e adesão ao plástico, uma vez que uma maior expressão das adesinas foi

observado somente entre os trofozoítos aderidos às células epiteliais vaginais. No entanto, o LPG parece ser importante tanto na citoaderência quanto na adesão ao plástico, uma vez que a oxidação do LPG reduziu de forma similar e de forma significativa a citoaderência e a adesão. Esses resultados indicam que o *T. vaginalis* possui um complexo mecanismo de citoaderência e adesão que garantirá o sucesso do parasitismo.

Palavras-chave: *Trichomonas vaginalis*, prevalência, incidência, genes normalizadores, adesão, citoaderência, DIU, anel vaginal.

ABSTRACT

Introduction: *Trichomonas vaginalis* is an extracellular protozoan and the etiologic agent of trichomonosis, the most prevalent sexually transmitted disease in the world. Cytoadherence has been the most studied pathogenic mechanism, however, there are few studies about adherence to plastic by *T. vaginalis* and to the best of our knowledge no study has evaluated the *T. vaginalis* adhesion to intrauterine device (IUD) and vaginal ring. **Objectives:** (i) to conduct a systematic review in order to determine both prevalence and incidence values of trichomonosis among general population from five-continent regions; (ii) to determine the stability from candidate reference genes for use in gene expression normalization; (iii) to evaluated the ability of *T. vaginalis* fresh isolates to adhere to plastic, host cells and contraceptive devices; (iv) to compare the mechanism involved in adhesion to plastic and cytoadherence processes. **Methods:** We conducted a systematic review of the literature following PRISMA guidelines. The transcripts of nine candidate reference genes were quantified using qRT-PCR under different cultivation conditions, and the stability of these genes was compared using the geNorm and NormFinder algorithms. Herein we investigated the adherence ability of *T. vaginalis* fresh clinical isolates to plastic, IUD and vaginal ring. The influence of LPG, cytoskeletal components of the parasite, surface molecules and the gene expression from four adhesins proteins were also investigated in the cytoadherence to human cells and in adhesion to plastic. **Results:** The systematic review showed that the most robust data were found in the US; in the other hand other regions have limited numbers of studies, thus the data obtained in this review point that the overall burden of trichomonosis in world as a whole is still unknown. In the validation of reference genes, the most stable genes were *α-tubulin*, *actin* and *DNATopII*, and, conversely, the widely used *T. vaginalis* reference genes *GAPDH* and *β-tubulin* were less stable. Of 32 *T. vaginalis* isolates studied here, 19 (59.37%), were able to adhere in polystyrene microplates. The *T. vaginalis* isolates TV-LACM6 and TV-LACM14, which were strong plastic-adherent, were also able to adhere to IUD and vaginal ring. Following chemical treatments, the *T. vaginalis* components, LPG, cytoskeletal proteins and other surface molecules, were involved in both adherence to plastic and cytoadherence. The gene expression level from four adhesins proteins were highest in trophozoites adhered in CEVs than in

trophozoites adhered to the abiotic surface (plastic). **Conclusions:** Few data about the numbers of prevalence and incidence of trichomonosis are available, consequently robust epidemiological studies of prevalence and incidences showing the disease ongoing are needed to guide priorities and define public health policies. Considering the validation of reference genes, this study is the first systematic exploration of *T. vaginalis* to identify optimal reference genes for qRT-PCR normalization under different culture conditions. This is valuable for future research on *T. vaginalis* gene expression studies. Finally, our results reaffirm the importance of *T. vaginalis* as an important problem among women in reproductive age, because the trophozoites were able to adhere in contraceptive devices and this fact could facilitate the parasite infection in the vaginal cavity. Moreover, our results indicate a possible difference between cytoadherence process and adhesion to plastic, subsequently the high level of adhesin protein expression were observed only in trophozoites attached to VECs. However, the LPG appears to be important in both processes, since the oxidation of LPG similarly and significantly reduced the adherence and cytoadherence. These results indicate that *T. vaginalis* presents multiple and complex mechanisms for attachment to host cells and to abiotic surfaces, ensuring successful parasitism.

Keywords: *Trichomonas vaginalis,* prevalence, incidence, housekeeping genes, adherence, cytoadherence, IUD, vaginal ring.

SUMÁRIO

PARTE I

I.1 INTRODUÇÃO

I.1.1 *Trichomonas vaginalis*

O protozoário flagelado *Trichomonas vaginalis* foi descrito por Alfred Donné em 1836, e o parasito pertence à família Trichomonadidae, ordem Trichomonadida, classe Parabasalia e filo Zoomastigina. (SCHWEBKE e BURGESS, 2004).

O *T. vaginalis* apresenta um único estágio de trofozoíto, característica comum a todos os tricomonadídeos. Os aspectos morfológicos dos trofozoítos são os seguintes: o tamanho de 9,7 µm de comprimento por 7,0 µm de largura; presença de cinco flagelos, sendo quatro anteriores e livres, o quinto é incorporado à membrana ondulante do parasito; o núcleo está localizado próximo à extremidade anterior; o axóstilo é uma estrutura rígida e hialina que se projeta a partir da pelta através do centro do organismo, prolongando-se até a extremidade posterior. (BENCHIMOL, 2004; HONIGBERG e BRUGEROLLE, 1990; PETRIN et al, 1998). A pelta está presente na região anterior do corpo celular, a qual desempenha funções de sustentação e divisão da célula. (BENCHIMOL, 2004).

O parasito em condições ideais de cultivo ou ambiente mantém a sua morfologia piriforme, elipsoide ou oval. Todavia, condições físico-químicas, tais como pH, temperatura, tensão de oxigênio e força iônica podem afetar a sua forma, compondo pseudocistos imóveis, devido à internalização dos flagelos. (PEREIRA-NEVES, RIBEIRO, BENCHIMOL, 2003; HONIGBERG e BRUGEROLLE, 1990). A alteração morfológica dos trofozoítos também ocorre quando eles se encontram em contato com as células epiteliais vaginais (CEVs), transformando-se do aspecto piriforme para ameboide. (ARROYO et al, 1993).

Os tricomonadídeos exibem uma organela incomum, o hidrogenossomo visto que não possuem mitocôndrias nem peroxissomos. Os hidrogenossomos são grânulos densos, distribuídos por todo o citoplasma e especialmente concentrados próximos ao axóstilo e à costa. (MÜLLER, 1990; MÜLLER, 1993; BENCHIMOL, 2009). Essas organelas apresentam funções metabólicas similares às mitocôndrias, pois, têm uma alta atividade enzimática e também estão envolvidos no metabolismo energético do parasito. (KULDA, 1999).

O protozoário *T. vaginalis* é um organismo anaeróbio facultativo e, em cultivo, cresce bem tanto na presença quanto na ausência de oxigênio em temperaturas entre 20 e 40ºC. Como fonte energética, o parasito utiliza os carboidratos glicose, frutose e maltose, não sendo capaz de utilizar, entretanto, sacarose e manose. Os açúcares são degradados tanto no citoplasma como nos hidrogenossomos, sob condições anaeróbicas ou aeróbicas, formando ácidos orgânicos. (LINDMARK e MÜLLER, 1973).

Em situações de privação de carboidratos, a obtenção de energia se dá por meio de aminoácidos ou proteínas digeridas. (PETRIN et al, 1998). O parasito é incapaz de sintetizar ácidos graxos e esterol, dependendo de fontes exógenas desses nutrientes, como meios de cultura contendo soro bovino. (BEACH et al, 1990; 1991). Além disso, o *T. vaginalis* não realiza síntese *de novo* de nucleotídeos púricos e pirimídicos. A aquisição de nucleosídeos ou de bases púricas e pirimídicas ocorre por um sistema de salvação simples, com uma rota de duas enzimas: a purina nucleosídeo fosforilase (PNP, do inglês *purine nucleoside phosphorylase*), a qual catalisa a interconversão entre bases e nucleosídeos púricos, e a purina nucleosídeo quinase (PNK, do inglês *purine nucleoside kinase*), que é capaz de converter nucleosídeos em nucleotídeos. (MUNAGALA e WANG, 2003).

Para o crescimento (além de purinas, pirimidinas e lipídeos) o parasito necessita de vitaminas e sais inorgânicos, pois o metabolismo fermentativo do protozoário não é um processo de alto rendimento. Sendo assim, o *T. vaginalis* precisa de um ambiente rico em nutrientes para sobreviver. (CUDMORE et al, 2004). A obtenção desses nutrientes é realizada, a partir das secreções vaginais ou por meio da fagocitose de células bacterianas ou até mesmo da fagocitose de células do hospedeiro. (HUGGINS e PETRI, 1981; PEREIRA-NEVES e BENCHIMOL, 2007). Dessa maneira, os nutrientes, macromoléculas essenciais, vitaminas, minerais e o soro bovino (que é outra importante fonte nutricional para o crescimento dos trofozoítos) devem ser fornecidos no meio de cultura para garantir o máximo de crescimento e multiplicação dos parasitos. (LINSTEAD, 1990).

I.1.2 Tricomonose

A tricomonose é uma doença sexualmente transmissível (DST) e, segundo a Organização Mundial da Saúde (OMS), é a DST não viral mais frequente no mundo. (WHO, 2012). O número de novos casos das DSTs curáveis (infecções causadas por Chlamydia trachomatis, Neisseria gonorrhoeae, Treponema pallidum e Trichomonas vaginalis) em adultos, entre 15 e 49 anos de idade, foi estimado em 498,9 milhões, sendo a incidência de T. vaginalis de 276,4 milhões. (WHO, 2012). O número estimado para a prevalência da tricomonose é muito maior, quando comparada à prevalência das demais DSTs avaliadas pela OMS. Uma estimativa de 187 milhões de casos foi feita para o *T. vaginalis*, seguida por 100,4 milhões para a *C. trachomatis*, de 36,4 milhões para *N. gonorrhoeae* e de 36,4 milhões para *T. pallidum.* (WHO, 2012).

Existem inúmeras limitações, no entanto, para a obtenção de números exatos da tricomonose: elevado número de casos sem sintomas clínicos; a baixa sensibilidade dos métodos diagnósticos utilizados dificulta o acompanhamento clínico da doença; a tricomonose não se encontra entre as doenças de notificação compulsória; e pacientes assintomáticos não são testados para a tricomonose em clínicas de DSTs. (PIPERAKI et al, 2010; MEITES et al 2013). Além disso, os estudos de prevalência de qualidade disponíveis são limitados. Tais eventos podem levar à subestimação dos números apresentados, indicando que a incidência e prevalência desta infecção podem ser mais elevadas. Consequentemente, os números, atualmente descritos, embora extremamente elevados, podem não representar a realidade sobre a tricomonose no mundo. (WHO, 2012; MILLER e NYIRJESY, 2011).

A infecção por *T. vaginalis,* em mulheres, apresenta um amplo espectro de manifestações clínicas, variando desde a apresentação assintomática até um estado de severa vaginite. (MILLER e NYIRJESY, 2011). A sintomatologia, na grande maioria das pacientes, é caracterizada por um corrimento tipicamente amarelado ou esverdeado, espumoso e mucopurulento. Além disso, podem ser observados sinais ou sintomas como irritação e prurido vulvar, pequenos pontos hemorrágicos na mucosa vaginal ou cervical, acompanhados de edema e eritema, o que confere uma aparência conhecida como *colpitis macularis* ou aspecto de morango, dor abdominal inferior e disúria. (MILLER

e NYIRJESY, 2011; PETRIN et al, 1998; SCHWEBKE e BURGESS, 2004). O elevado número de casos assintomáticos na tricomonose, em torno de 80%, contribui para torná-la uma doença de alto risco, uma vez que casos não diagnosticados podem levar as graves complicações, tais como baixo peso em recém-nascidos, partos prematuros, câncer cervical e de próstata. Além disso, é importante ressaltar que casos assintomáticos também estão associados à aquisição e transmissão do HIV. (POOLE e MCCLELLAND, 2013).

A infecção nos homens, na maioria dos casos, é auto-limitada e assintomática, sendo assim, é fundamental o tratamento concomitante dos parceiros das mulheres infectadas, como medida para evitar a reinfecção. (CUDMORE et al, 2004). Os sintomas descritos nos homens são corrimento uretral, prurido, disúria, aumento da frequência urinária e dor abdominal inferior. (MILLER e NYIRJESY, 2011). A presença de cátions de zinco nas secreções prostáticas, os quais são citotóxicos ao parasito e a natureza oxidativa do trato genital masculino justificam a ocorrência transitória desta infecção nos homens. (CUDMORE et al, 2004). Nos homens, podem ocorrer, também, complicações devido à tricomonose, como prostatite, epididimite, balanopostite e infertilidade. (CUDMORE et al, 2004; KRIEGER et al, 1993). Além disso, Sutcliffe et al (2012) demonstraram que existe uma associação positiva entre a infecção por *T. vaginalis* e o câncer de próstata.

A tricomonose é uma importante patologia para as mulheres em idade reprodutiva, uma vez que pode causar sérias complicações, como infertilidade, predisposição ao câncer cervical, doença inflamatória pélvica, parto prematuro e baixo peso de recém-nascidos. (KLEBANOFF et al, 2001; GOLDSTEIN et al, 1993; VIIKKI et al, 2000; COTCH et al, 1991; 1997). Além disso, o *T. vaginalis* é considerado um agente facilitador da transmissão e aquisição do vírus da imunodeficiência adquirida (HIV). (QUINLIVAN et al, 2012; SORVILLO et al, 2001; VAN DER POL *et al*, 2008).

O tratamento preconizado para a tricomonose baseia-se no uso dos 5-nitroimidazóis - metronidazol e tinidazol, únicas opções terapêuticas aprovadas pelo FDA (do inglês: *Food Drug Administration,*), USA sendo o metronidazol o fármaco de escolha para esta doença. (HELMS et al, 2008). No entanto, a utilização destes fármacos apresenta limitações, tais como efeitos adversos, carcinogênicos e teratogênicos, embora

com baixo risco, devido à capacidade do fármaco de atravessar a barreira placentária. (ROSA et al, 1987; BEARD et al, 1979). A maioria das infecções é curada por uma dose única de metronidazol, todavia, a resistência de isolados de *T. vaginalis* ao fármaco já foi descrita há mais de 30 anos. (LOSSICK et al, 1986; MEINGASSNER e THURNER, 1979). De acordo com Perez et al (2001); Schmid et al (2001); Schwebke e Barrientes (2006) os valores de prevalência para os isolados resistentes são estimados entre 2,5% e 9,6%. Existe pouca informação, entretanto, sobre a resistência aos 5-nitroimidazóis entre isolados clínicos de *T. vaginalis*. Não há sistema de vigilância para detectar a resistência, podendo este número estar subestimado, uma vez que ocorrem cerca de 276,4 milhões de novos casos da doença a cada ano. (WHO, 2001).

O metronidazol é um pró-fármaco, e o grupamento nitro presente na molécula, precisa ser reduzido para exercer a sua toxicidade. O fármaco atravessa a membrana do parasito e as organelas por difusão passiva. (CUDMORE et al, 2004). O hidrogenossomo tem um importante papel na ativação da droga, uma vez que a sua maior parte será ativada nesta organela, pela ação da enzima piruvato:ferredoxina oxidoredutase (PFOR). Como resultado serão formados intermediários radicais-nitro citotóxicos, os quais induzem quebras cromossômicas, conduzindo o protozoário à morte. (KULDA 1999; DUNNE et al, 2003; CUDMORE et al, 2004; LAND et al, 2004).

I.1.3 Patogenicidade do *T. vaginalis*

Os mecanismos envolvidos na infecção e na patogênese da tricomonose baseiam-se na complexa interação parasito-hospedeiro, na qual estão envolvidos componentes associados à superfície celular do parasito e às CEVs do hospedeiro, assim como substâncias solúveis encontradas na secreção vaginal e uretral. (LEHKER e ALDERETE, 2000; BRAVO et al, 2010).

O *T. vaginalis* é um parasito exclusivo do trato-urogenital humano, sendo o muco que recobre as CEVs a primeira barreira imposta pelo hospedeiro. A mucina é uma proteína que compõe o muco e tem elevado peso molecular, é altamente glicosilada apresentando propriedades de gel. A mucina é, portanto, uma eficiente barreira física que limita o acesso do parasito às camadas mais profundas do epitélio. (GERKEN,

1993). A ligação dos trofozoítos ao muco é a primeira etapa para o parasito manter-se no sítio de infecção, seguida pela secreção de mucinases (enzimas que solubilizam a mucina), as quais permitem que os trofozoítos penetrem na matriz solubilizada pela movimentação flagelar, até alcançarem as CEVs. (LEHKER e SWEENEY, 1999).

O *T. vaginalis* é um patógeno extracelular e a citoaderência, ou seja, a aderência às CEVs do hospedeiro é fundamental para o processo de colonização e instalação da infecção. (ALDERETE et al, 1995; BASTIDA-CORCUERA et al, 2005). Trata-se de um processo complexo que envolve as proteínas de superfície, glicoconjugados e proteínas do citoesqueleto. (FIGUEROA-ÂNGULO et al, 2012).

As adesinas de superfícies são proteínas exaustivamente estudadas como as principais moléculas envolvidas na adesão dos trofozoítos às células hospedeiras. (ENGBRING e ALDERETE, 1998; KUCKNOOR et al, 2005; MORENO-BRITO et al, 2005). Não há descrição de receptores, no entanto, na superfície das CEVs, para fazer a ligação parasito-hospedeiro com estas moléculas. (BASTIDA-CORCUERA et al 2005; OKUMURA, BAUM, JOHNSON, 2008). De acordo com Alderete e Garza, (1985), Engbring e Alderete (1998), Kucknoor et al, (2005) e Moreno-Brito et al (2005) existem cinco adesinas denominadas AP23, AP33, AP51, AP65 e AP120 (AP, do inglês: *adhesin protein* e peso molecular), as quais são expressas por famílias multigenes e foram identificadas e caracterizadas como proteínas multifuncionais, com funções dependentes da localização, considerando que: (a) atuam como adesinas durante a interação com as CEVs; (b) apresentam atividade metabólica no hidrogenossomo e citoplasma (pois apresentam grande homologia com as enzimas hidrogenossomais PFOR, enzima málica (AP65) e com subunidades α e β-succinil-CoA sintetase (AP33 e AP51)); (c) podem mediar a ligação à hemoglobina para a aquisição de ferro, principalmente durante o ciclo menstrual devido à grande disponibilidade de eritrócitos, visto que o ferro é um nutriente essencial para o parasito e (d) são consideradas moléculas mimetizadoras (ALDERETE et al, 2001), envolvidas na evasão imune, portanto apresentam grande identidade com enzimas do hospedeiro, exceto a AP120. (FIGUEROA-ÂNGULO et al, 2012; ALDERETE et al, 1995; ENGBRING e ALDERETE, 1998; MORENO-BRITO et al, 2005).

O *T. vaginalis* é coberto por um denso glicocálice, o qual tem como principal componente o lipofosfoglicano (LPG), que é o mais profuso polissacarídeo de superfície. (BASTIDA-CORCUERA et al, 2005). A existência de LPG na superfície celular do *T. vaginalis* foi completamente definida por Singh em 1994. No entanto, a estrutura detalhada do polissacarídeo ainda é desconhecida. Análises na composição dos monossacarídeos demonstraram, porém, que a galactose e a glicosamina são os mais abundantes resíduos de monossacarídeos presentes na estrutura do LPG. (SINGH, 1993). O LPG tem alta concentração na superfície do parasito e constitui-se numa molécula complexa, possuindo uma âncora de fosfoceramida inositol e um núcleo glicano fosforilado, composto de 50 a 54 resíduos de monossacarídeos, com uma composição diferente de outros parasitos. (SINGH, 1994).

Segundo Bastida-Corcuera et al (2005), esses carboidratos de superfície são pouco explorados quanto ao seu papel na adesão. Entretanto, ao expor os trofozoítos de *T. vaginalis* ao periodato, que é um forte oxidante de polissacarídeos de superfície (GILBERT et al, 2000), às enzimas que digerem o glicocálice (MIRHAGHANI et al 1998) ou a outros açúcares que competem para a ligação (GOLD e OFEK, 1992), houve significativa redução na capacidade de adesão às células mamíferas. Em um elegante estudo realizado por Bastida-Corcuera et al (2005) foram obtidos isolados de *T. vaginalis* com LPG mutantes por meio de modificações na composição dos açúcares. Esses mutantes exibiram uma significativa redução na adesão e na citotoxicidade às células ectocervicais humanas, quando comparados com o isolado selvagem. Okumura e colaboradores em 2008, demonstraram que a galactina 1(Gal-1), que pertence à família das lectinas, é expresso pelas células do epitélio cervical e liga-se ao LPG do *T. vaginalis*, através de uma ligação dependente de carboidrato, ou seja, de galactose. (OKUMURA, BAUM, JOHNSON, 2008).

Além das adesinas e do LPG, os receptores da matriz extracelular e as cisteína proteinases (CPs), tais como TvCP30 e TvCP62, localizadas na superfície do parasito, também participam no mecanismo de citoaderência. (FIGUEROA-ÂNGULO et al, 2012). O parasito também pode utilizar componentes da matriz extracelular e da membrana basal, como a fibronectina e a laminina para o estabelecimento da colonização e da infecção persistente (CROUCH e ALDERETE, 1999). Considerando

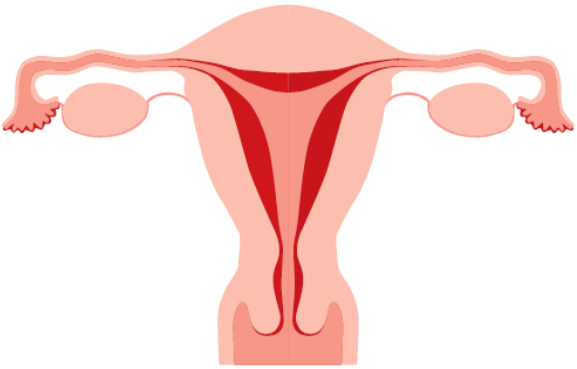
a indução da expressão de genes que codificam a fibronectina, a qual medeia a interação dos parasitos às CEVs, pode-se considerar esse mecanismo como uma via alternativa às adesinas para a colonização do epitélio vaginal (KUCKNOOR et al, 2005). A citoaderência permite que um segundo processo ocorra, a citotoxicidade, a qual envolve eventos em série que resultam na citólise, fagocitose e desintegração de monocamadas de células. Produtos secretados e liberados pelo *T. vaginalis*, como glicosidases e CDF (do inglês, *Cell-Detaching Factor*) são altamente citotóxicos às CEVs (FIGUEROA-ÂNGULO et al, 2012).

O parasito, após colonizar a mucosa vaginal, necessita de nutrientes e precisa evadir a resposta imune do hospedeiro. Para isso lança mão da fagocitose, que é o principal mecanismo de aquisição de nutrientes e, apesar deste mecanismo não estar totalmente elucidado, dois comportamentos diferentes são observados: a formação de pseudópodes em direção à célula alvo; e um processo de afundamento, sem extensão da membrana. (FIGUEROA-ÂNGULO et al, 2012). Os trofozoítos são capazes de ingerir e degradar com eficiência bacilos de Döderlein, CEVs e células epiteliais cervicais, leucócitos, eritrócitos, leveduras, espermatozoides e células prostáticas, adquirindo, dessa forma, ferro, lipídeos, nucleotídeos e nucleosídeos, dentre outros nutrientes importantes para o seu metabolismo (PEREIRA-NEVES et al, 2007; FIGUEROA-ÂNGULOet al, 2012). Os eritrócitos são uma das vias de aquisição de nutrientes como lipídios e ferro, e a hemólise decorre da interação entre os trofozoítos e os eritrócitos (Fiori et al, 1993). As CPs também têm sido consideradas prováveis fatores líticos envolvidos na hemólise e sua atividade está relacionada à aderência dos parasitos às CEVs. (ARROYO e ALDERETE, 1989).

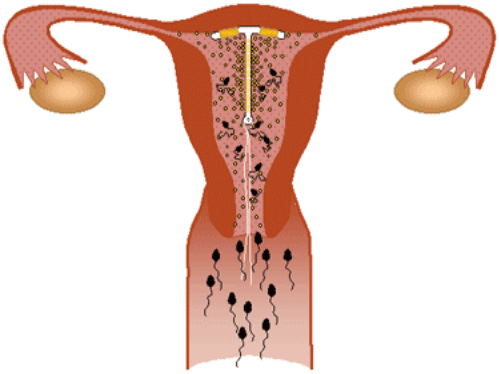
Até o presente momento, pouco se avaliou sobre a adesão do *T. vaginalis* a superfícies abióticas, a adesão do *T. vaginalis* ao vidro e ao poliestireno foi descrita em 1975, por Cappuccinelli e Varesio e, em 1993 por Gold. Os carboidratos de superfície celular do parasito são considerados o principal mecanismo envolvido na aderência a superfícies de vidro e ao plástico. (PETRIN et al, 1998; BASTIDA-CORCUERA et al, 2005).

I.1.4 Métodos contraceptivos: dispositivo intrauterino (DIU) e anel vaginal

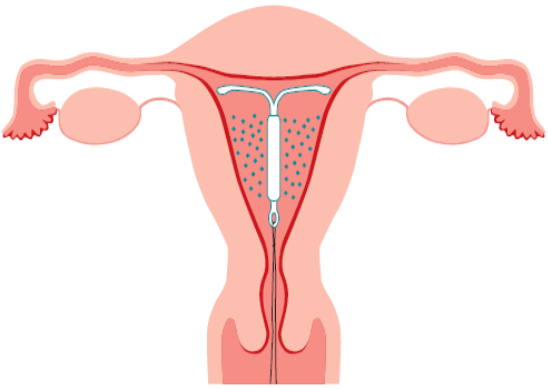
O dispositivo intrauterino (DIU) é um dispositivo de plástico recoberto com cobre e/ou hormônio inserido na cavidade uterina (Figura 1). É considerado o segundo método mais prevalente de planejamento familiar no mundo (13,6%) após a esterilização feminina (20,5%). Os DIUs têm uma durabilidade que varia de 5 a 10 anos. (MORRISON et al, 2009).



Cavidade uterina antes da inserção do



Cavidade uterina com DIU de cobre (cobre sendo liberado)



Cavidade uterina com DIU hormonal (levonorgestrel sendo

Figura 1 - Cavidade uterina e posição do DIU imagens retiradas de Bayer Health Care. (Disponível em:<http://www.bayerhealthcare.com.br/>. Acesso em 11 de abril de 2015)

O primeiro DIU foi utilizado no ano de 1961. Os primeiros DIUs eram feitos de materiais plásticos inertes e foram substituídos por produtos que liberam cobre ou levonorgestrel, modificações estas que aumentaram substancialmente a sua já alta

eficácia. Os índices de eficácia são semelhantes aos dos contraceptivos orais, ou seja, 0,1% de falha. (The ESHRE CAPRI WORKSHOP GROUP, 2008). Os DIUs existentes que contêm cobre são constituídos de polietileno (PE) ou polipropileno (PP) e uma grande quantidade de cobre. Compreendem um dos métodos mais utilizados na contracepção, principalmente devido as suas vantagens que incluem a elevada eficácia e a longa duração, o baixo custo e a alta segurança, além da reversibilidade. (XIA et al, 2007).

Estudos têm demonstrado que a inserção do DIU pode modificar, de forma significativa, a microbiota vaginal, a qual desempenha função como primeira barreira imune do trato genital pode proteger contra doenças infecciosas. (ELHAG, BAHAR, MUBARAK, 1988; TIBALDI et al, 2009; DO LAGO et al, 2003). Além disso, a inserção do dispositivo perturba a barreira de proteção do muco cervical e seu apêndice (cauda acoplada a ele) (Figura 1) pode servir como um caminho facilitador para a migração de patógenos vaginais em direção à cavidade uterina. (ELHAG, BAHAR, MUBARAK, 1988). Assim, as usuárias de DIU podem estar substancialmente mais suscetíveis a patologias do trato urogenital. Alguns estudos têm demonstrado elevada frequência de DSTs em pacientes usuárias de DIU, incluindo tricomonose. (HAUKKAMAA et al, 1986; ROY, 1991; CERUTI et al, 1994; KAZEROONI e MOSALAEE, 2002). No estudo de Kazerooni e Mosalee (2002) *T. vaginalis* foi significativamente mais prevalente em usuárias de DIU do que em outros métodos contraceptivos. Nasir e colaboradores (2005) detectaram uma taxa de prevalência de tricomonose de 34% em usuárias de DIU, e de somente 5% em não usuárias.

Além do DIU, o anel vaginal também tem sido utilizado como método contraceptivo desde 1960, com diversos formatos e formulações. (WIEDER & PATTIMAKIEL, 2010). O anel vaginal NuvaRing® (Organon, Kenilworth, New Jersey), foi aprovado pelo FDA/USA em 1991, e é produzido utilizando um copolímero de etileno vinil acetato. Trata-se de um anel plástico não biodegradável, sem látex, flexível e incolor com diâmetro externo de 54 mm e espessura de 4 mm (Figura 2A). Cada anel contém 2,7 mg de etinilestradiol e 11,7 mg de etonogestrel. Após a colocação na vagina, será liberado em média 0,015 mg por dia de etinilestradiol e 0,120 mg por dia de etonogestrel num período de 3 semanas (Wieder & Pattimakiel, 2010). O anel

vaginal fica localizado na porção alta da vagina e a própria paciente faz a colocação e a retirada do dispositivo (Figura 2B).

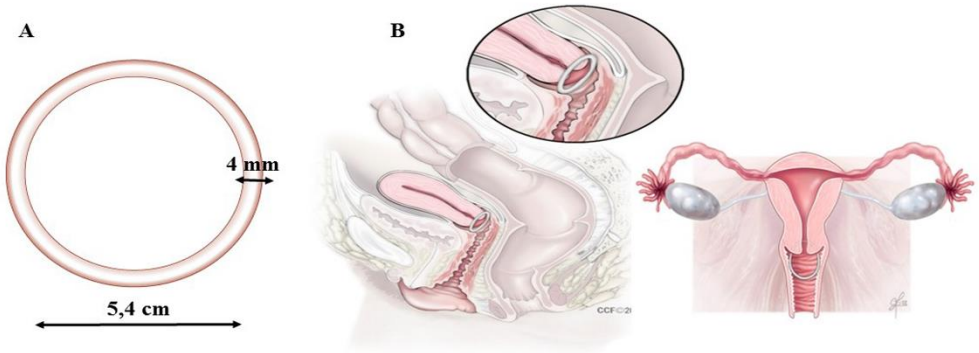


Figura 2: Anel vaginal e posição do anel vaginal na vagina. (A) Tamanho do anel vaginal retirado de: http://www.anticoncepcao.org.br/html/manual/corpo/anel_vaginal/index.asp. Acesso em: 06 de abril de 2015. (B) Posição do anel na vagina. Retirado de Wieder & Pattimakiel (2010). Examining the efficacy, safety, and patient acceptability of the combined contraceptive vaginal ring (NuvaRing®). International Journal of Women's Health 2: 401–409.

I.1.5 O genoma do *T. vaginalis* e estudos de expressão gênica

O genoma do *T. vaginalis* foi publicado em 2007 por Carlton e colaboradores. A sequência genômica completa, descrita para o isolado *T. vaginalis* G3 tem, aproximadamente, 160 Mb, distribuídos em apenas 6 cromossomos, sendo que ao menos 65% do genoma é repetido. Encontram-se nele um conjunto de ~ 60.000 proteínas que são codificadas por genes. Foram descritos também aproximadamente 1.100 genes de RNA ribossomal e ao menos 14.390 sequências de RNA viral ou elementos de transposição (*transposons*) em fase de leitura aberta (do inglês: *open reading frames* ORFs). (CARLTON et al, 2007). Ao considerar as características singulares do genoma do *T. vaginalis* Carlton e colaboradores, o classificaram como o organismo com maior capacidade de codificar proteínas entre os eucariotos, uma vez que, surpreendentemente, o material genético do *T. vaginalis* é maior do que o genoma de outros protozoários como, por exemplo, *Entamoeba (*~ 20 Mb), *Plasmodium* (~ 25 Mb) e *Toxoplasma* (~ 63 Mb). (CONRAD et al 2013). A descrição do genoma completo do *T. vaginalis*, assim como a sua disponibilidade em uma base de dados livre como o Trichdb (http://www.trichdb.org/trichdb/), facilitou estudos de biologia molecular básica,

especialmente aqueles que envolvem a análise da expressão de genes em *T. vaginalis.* **(**BRADIC et al, 2014).

A reação de transcrição reversa, seguida da reação da polimerase em cadeia de forma quantitativa - qRT-PCR (do inglês: *quantitative reverse transcriptase PCR*) é um método amplamente utilizado, constituindo-se em uma robusta ferramenta, devido a sua rapidez, sensibilidade, especificidade e, sobretudo pela medida precisa dos níveis de mRNA. (VANGUILDER, VRANA, FREEMAN, 2008; BRADIC et al, 2014; NAPOLITANO et al, 2014). Além disso, existem genes que são expressos em baixos níveis. Dessa forma, somente utilizando-se o qRT-PCR é possível determinar o número de cópias do mRNA. (NAPOLITANO et al, 2014).

No entanto, inúmeras são as variáveis técnicas, inerentes a esta metodologia, as quais podem levar a uma interpretação errônea dos dados. Entre as principais limitações descritas encontram-se a qualidade e a quantidade de RNA acrescentado na reação de PCR, erros de transcrição reversa e erros de pipetagem. (KOZERA e RAPACZ, 2013). Para minimizar ou evitar as influências dessas variáveis emprega-se a normalização do gene estudado com um *housekeeping gene* ou um gene normalizador. Esta abordagem permite regular variações não biológicas, desde que ambos os genes (teste e normalizador) sejam expostos às mesmas variações técnicas. (REDDY et al, 2013; KOZERA e RAPACZ, 2013). Um gene de referência ideal deve ter uma mínima variação em seu nível de expressão constitutiva frente às diferentes condições experimentais. Muitos genes denominados como housekeeping apresentam, todavia, instabilidade em seu nível de expressão constitutivo. (KOZERA e RAPACZ, 2013; HUGGETT et al, 2005; MAROUFI et al, 2010). Acredita-se que isso ocorra pelo fato da seleção do gene de referência basear-se no uso tradicional. Por exemplo, os genes gliceraldeído-3-fosfato desidrogenase (GAPDH), fator de elongação, actina e alfa-tubulina são considerados genes de referência razoáveis, devido ao seu nível constitutivo de expressão, condição suportada somente por estudos qualitativos, isto é, análises que utilizam a metodologia de Northern blotting. (VANDESOMPELE et al, 2002; DERVEAUX et al, 2010; KOZERA e RAPACZ, 2013). Neste contexto, antes da utilização de um gene como referência é fundamental que a sua expressão constitutiva seja avaliada, ou seja, a sua estabilidade. (VANDESOMPELE et al, 2002).

Ao analisar o referencial teórico apresentado, é fundamental considerar que a tricomonose representa um complexo problema de Saúde Pública, devido ao expressivo aumento de casos a cada ano e às graves consequências provocadas pela doença, especialmente no período gestacional. O *T. vaginalis* ocasiona partos prematuros, baixo peso ao nascimento e a infecção está fortemente relacionada ao aumento da transmissão do HIV, à infertilidade e à predisposição ao câncer cervical e de próstata. Atualmente, os fármacos de escolha para o tratamento da tricomonose limitam-se aos pertencentes à classe dos 5-nitroimidazois, sendo somente dois (metronidazol e tinidazol) aprovados para uso pelo FDA/USA. Nesse contexto, o tratamento torna-se restrito, apresentando diversas limitações relacionadas aos efeitos adversos e à resistência dos isolados clínicos de *T. vaginalis* aos fármacos de escolha. Considerando-se tal problemática, é evidente a necessidade de investigação dos aspectos biológicos do *T. vaginalis*, incluindo as suas propriedades bioquímicas, os mecanismos moleculares e os fatores de virulência, que são fundamentais para o entendimento da biologia e da patogênese desse protozoário. Assim, ao analisar (I) o impacto da tricomonose na saúde pública, (II) o número de casos negligenciados por falhas no diagnóstico ou no tratamento, (III) a necessidade de maior compreensão sobre os mecanismos moleculares que regem a relação parasito-hospedeiro, (IV) o limitado arsenal terapêutico disponível e o crescente número de isolados resistentes aos fármacos disponíveis, e por fim, (V) a elucidação de mecanismos de adesão que poderão apontar, tanto para novos alvos terapêuticos, quanto para diagnóstico, foram estabelecidos os objetivos desse estudo.

I.2 OBJETIVOS

I.2.1 Objetivo Geral

Estudar a epidemiologia da tricomonose e analisar os mecanismos envolvidos na adesão e citoaderência de *T. vaginalis* através da avaliação funcional de moléculas de superfície e expressão de adesinas após validação de gene de referência para estudos de expressão gênica.

I.2.2 Objetivos Específicos

a) Realizar uma revisão sistemática para avaliar as estimativas da prevalência e da incidência da tricomonose no mundo;
b) Validar genes de referência para uso nos experimentos de avaliação de expressão gênica em *T. vaginalis*;
c) Investigar a capacidade de adesão ao plástico de isolados ATCC e frescos de *T. vaginalis;*
d) Avaliar a capacidade de adesão ao dispositivo intrauterino (DIU) e ao anel vaginal de isolados ATCC e frescos de *T. vaginalis;*
e) Avaliar o envolvimento do lipofosfoglicano e de outras moléculas de superfície do *T. vaginalis*, bem como dos componentes do citoesqueleto do parasito nos processos de citoaderência e de adesão às superfícies abióticas;
f) Avaliar o nível de expressão das adesinas de superfície em *T. vaginalis* aderidos em superfície abiótica e em células epiteliais vaginais;
g) Avaliar se existem semelhanças entre os mecanismos de citoaderência e de adesão às superfícies abióticas.

PARTE II

II.1 ARTIGOS CIENTÍFICOS

II.1.1 Artigo 1: Trichomonosis: a systematic review of the global prevalence and incidence. Odelta dos Santos, Graziela de Vargas Rigo, Alexandre José Macedo and Tiana Tasca.

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Trichomonosis: a systematic review of the global prevalence and incidence

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Short title

Trichomonas vaginalis prevalence and incidence

Abstract

Trichomonosis, caused by *Trichomonas vaginalis*, is the *most* common non-viral sexually transmitted disease in world and may induce serious health complications. We conducted a systematic review of the literature following PRISMA guidelines to determine the burden of trichomonosis in five world-regions: America, Asia, Africa, Europe, and Oceania. We found 23 articles with original prevalence or incidence data; highest prevalence estimates was 17.6% among women and men from remote Australian Aboriginal population and the lowest rates 0.85% was found among women with ages 15-49 yr from Grenada. Incidence estimates variation were from 22.1 cases per 100 person-year among women in the US to 3.6 cases per 100 person-year among men in Australian Aboriginal populations. The most robust data were found in the US; in the other hand other regions have limited numbers of studies. The significant associated risk factors included: black race, African American or Indigenous, high number of sexual partners, lack of condom use, early age of first sexual intercourse, use of alcohol, and cigarette or marijuana smoking. Further research is needed to determine the true current burden of trichomonosis in most of the world region, since data obtained in this review point that the overall burden of trichomonosis in world as a whole is still unknown.

Keywords: systematic review, prevalence, incidence, trichomonosis, *T. vaginalis*.

Introduction

The flagellated protozoan parasite *Trichomonas vaginalis* is the etiologic agent of trichomonosis, the most common non-viral sexually transmitted disease (STD) in world.[1] *Trichomonas vaginalis* has been associated with predisposition to cervical cancer, and pelvic inflammatory disease. Furthermore, this parasite is an important problem among women in reproductive age, because it can contribute to premature rupture of membranes during pregnancy, preterm delivery and low birth weight, besides leading to infertility and increased infant mortality.[2] Moreover, *T. vaginalis* has been associated to aggressive prostate cancers.[3] In addition, trichomonosis is a co-factor for transmission and acquisition of human immunodeficiency virus (HIV).[4]

Trichomonosis presents global prevalence and incidence values higher than other curable sexually transmitted infections, according to the estimate of World Health Organization (WHO) performed in 2008 and published in 2012.[1] At that time, it was estimate that the prevalence number of adults with *T. vaginalis* infection was 187.0 million. Considering 498.9 million of new cases of the four sexually transmitted infections (STIs) among adults, 276.4 million were *T. vaginalis* infection. Both values, prevalence and incidence, were highest for *T. vaginalis* infection than other sexually transmitted infections, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Treponema pallidum* (syphilis).[1]

According to the US Center for Disease Control and Prevention (CDC) (Atlanta, Georgia, US) *T. vaginalis* belongs to the group of five neglected parasitic infections (NPIs), together with Chagas disease, cysticercosis, toxocariasis, and toxoplasmosis. This classification is based on the number of people infected, severity of the illnesses, and ability to prevent and treat it, as well as, the low attention has been dedicate to their surveillance.[5] Each year 1.1 million people are newly infected with *T. vaginalis* in the United States (US); consequently, it is a major cause of infertility and preterm labor and low birth weight, and these data represent the estimates of burden and impact of trichomonosis in US.[5] The CDC points to 3.7 million people infected with this parasite in the US. In the same way, a previous published study affirms that *T. vaginalis* has received much less consideration than other sexually transmitted diseases, being

classified as neglected disease in the US.[6] Despite the risks associate with *T. vaginalis* infection, trichomonosis is not a reportable disease and often it is not included in routine testing, constituting an important global health burden. Unfortunately, although estimates of more than 180 million *T. vaginalis* infection per year worldwide performed in 2008,[1] very little is known about the trichomonosis rates in other world region than US. Considering that the global estimative of total *T. vaginalis* infection was evaluated seven years ago, the current burden of trichomonosis in world is unknown. We conducted a systematic review in order to determine both prevalence and incidence values of trichomonosis among general population from five-continent regions. In addition, we attempt to identify needed areas of future research.

Materials and Methods

The systematic review was conducted according to the PRISMA 2009 guidelines.[7]

Search strategy and selection criteria

The search was performed using PubMed databases and restricted to articles published between January 1st 2009 to January 1st 2015, in English language. We started our limited search in 2009 because the WHO published in 2012 a global estimative of curable sexually transmitted infections until 2008. The search terms included were the following: "trichomonosis prevalence"; trichomonosis incidence" and "prevalence" or "incidence" plus the region (Americas, South-East Asian, European, Eastern Mediterranean and Western Pacific, the same regions used by WHO in 2012). Searches were also performed using disease name variation as Medical Subject Headings (MeSH): *Trichomonas* vaginitis"; "*Trichomonas* infection" and "*Trichomonas* urethritis". The articles that described a different disease or about other *Trichomonas* disease topic, and those about *Trichomonas* in animals and duplicate results were excluded from the abstract review. We also excluded studies that focused in HIV-infected, pregnant women, sex workers, drug users, child victims of sexual assault, victims of sexual assault, people incarcerated, and non prevalence or non incidence data.

The articles that had in title the words “prevalence or incidence of sexually transmitted disease” or “frequency or occurrence of sexually transmitted infection” even with the absence of “trichomonosis or *Trichomonas vaginalis”* were also included in the abstract review. Eligible studies included original data on both prevalence and incidence. When the abstract did not offer sufficient information for inclusion or exclusion, the full-text was analyzed.

The search was performed by two independent reviewers based on the search protocol (Supplemental material 1). Discussion and consensus between reviewers were used to resolve the discrepancies found in the studies selection.

Data Extraction

Documents in the full-text review were classified as following: 1) original prevalence; 2) original incidence and 3) five continent origins (America including American United States; Europe; Asia; Africa, and Oceania). The articles were excluded in full-text review if they used inappropriate methodological approaches and inadequate statistical analysis. The full-texts were also independently revised by two reviewers; figures, tables or text containing original prevalence or incidence as well as descriptions of population affected and reported risk factors for disease were analyzed.

Results

We found 23 articles with original prevalence or incidence for trichomonosis (Figure 1).

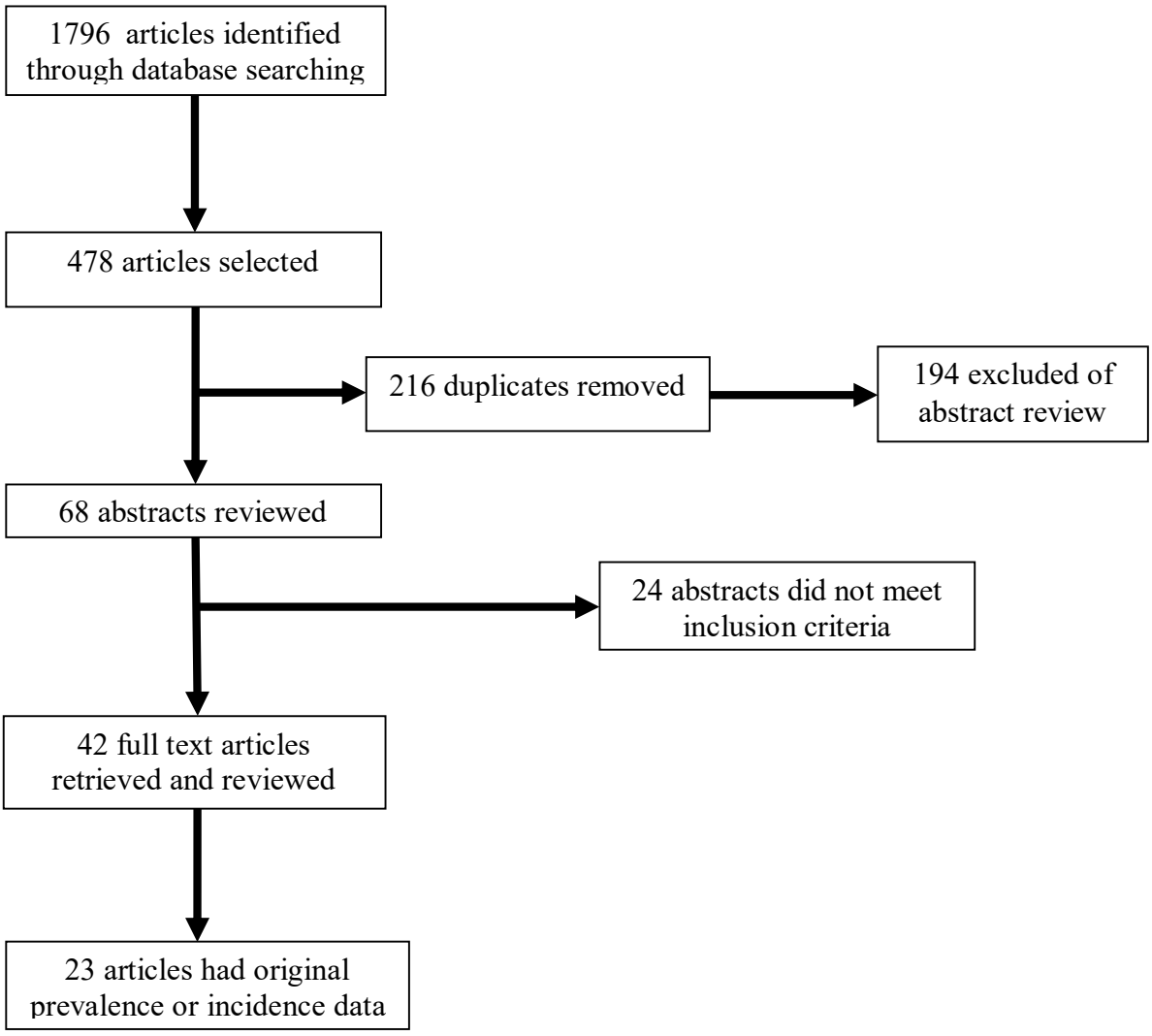

Figure 1. Systematic review process.

Among all five-world regions grouped, the America region had highest number of studies, with 14 articles found (Figure 2). [8-21]

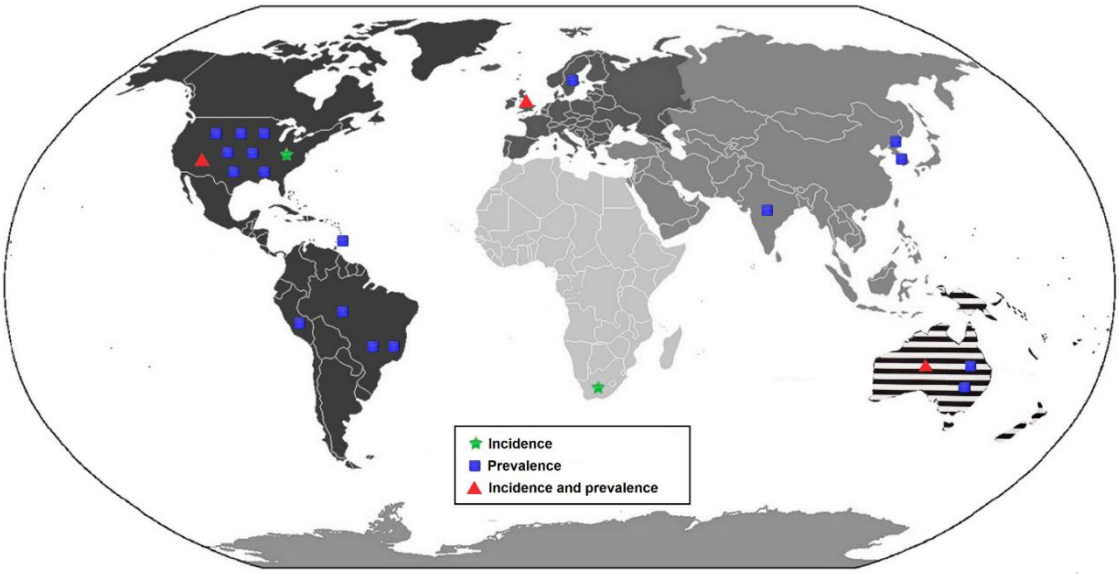

Figure 2. Geographical distribution of trichomonosis prevalence and incidence estimates in the five regions of the world.

Among these articles, nine reported data in the US, 8-16 three in Brazil,17-19 one in Peru, 20 and one in Grenada 21. The Asian region had three studies with original prevalence estimates, two from South Korea 22, 23 and one from India. 24 In Oceania, only three articles in Australia were found. 25 - 27 In the European region two articles were found, one in United Kingdom (UK) 28 and one in the Nordic countries. 29 In the African region it was found the lowest number of articles, only one article reported data in South Africa (Figure 2 and Table 1). [30]

Table 1 summarizes the prevalence and incidence of trichomonosis in the five world regions, 18 articles reported only prevalence data, and three articles reported both prevalence and incidence data and two articles reported only incidence data (Figure 2). Thus, the global prevalence estimates a range from 17.6% for women and men from remote Australian Aboriginal population[27] to 0.85% among women with ages 15-49 yr from Grenada, [21] that is an island country located in the southeastern Caribbean Sea. The original incidence data were reported in five articles. Of these articles, two reported data in America and one in Africa, Australia and Europe. Incidence estimates ranged from 22.1 cases per 100 person-year among women [10] in data for US to 3.6/100 person-years among men for Australian Aboriginal populations.[27]

Table 1 Summary of *T. vaginalis* prevalence and estimates in published literature.

Continent	Country	Location	Year	Prevalence	Incidence	*N*	Demographics	Article
America								
	US	Countrywide	2001-2004	3.2%		3,648	*NHANES data, women civilian and non-institutionalize, ages 14-49.	Allsworth et al 2009
	US	Countrywide	2003–2004	2.5%		838	*NHANES, female adoslescents, ages 14-19.	Forhan et al 2009
	US	Atlanta, GA.	2001-2005	14.4%	22.1 cases per 100 person-years	467	Women sexually active, nonpregnant, human immunodeficiency virus-seronegative, ages 13-19.	Krashin et al 2010
	US	Baltimore (Maryland), Maryland (outside Baltimore), District of Columbia (DC), West Virginia, selected of Illinois, and Denver (Colorado)	2006-2009	10%		501	Men, volunteers in internet-based screening,ages 21-30.	Chai et al 2010
	US	Maryland, the District of Columbia, West Virginia, selected counties in Illinois, Denver Colorado and Alaska.	2006-2012	3.7%;		1699	Sexually active men aged ≥14 years, recruited by an educational internet program.	Gaydos et al 2013
	US	St. Louis área	2007-2011	4.9%		8347	**CHOICE data, women not currently using a method of contraception, ages 14–45.	McNicholas et al 2013
	US	21 states	2010	8.7%		7,593	Women who were with or without symptoms, ages 18-89.	Ginocchio et al 2012
	US	Alabama, California, Colorado, New York, Pennsylvania and Washington	2010-2011	26.2% and 6.5%		59,176	All women patients visiting geographically diverse, publicly funded, urban STD clinics participating in the STD Surveillance Network (SSuN) established by the CDC in 2005, ages 20-29.	Meites et al 2013
	US	Atlanta, GA	2005-2007		20.0%	605	African American adolescent girls aged 14 to 20 years were recruited from reproductive health clinics to participate in an HIV/STI prevention trial.	Swartzendruber et al 2014
	Brazil	São Paulo	2004-2005	3.2%		787	Women attending in primary care health service located in the metropolitan region of São Paulo, ages 18-40.	Luppi et al 2011
	Brazil	Uberlândia, Minas Gerais.	2009-2010	2.6%		742	Women attending gynecology units of public health centers, ages 25-45.	Grama et al 2013
	Brazil	Coari, Amazonas	2010	12.7%		361	Women registered for health care in 10 Basic Healthcare Units, ages 18-78.	Rocha et al 2014
	Peru	Lima, Trujillo, and Chiclayo	2003-2005	9.1%		308	Women from 20 low-income communities in coastal Peru, ages 18-41.	Leon et al 2009
	Grenada	Grenada	2009-2011	0.85%		2677	Women between the ages of 15 and 49 years.	Brooks-Smith-Lowe and Rodrigo 2013
Africa								
	South Africa	Durban	2003-2006	6.5%	8.6/100 women-years	1485	Women participating in a phase III vaginal diaphragm trial.	Naidoo and Wand 2013
Europe								

	Nordic countries	Denmark, Norway, Iceland, and Sweden	2004-2005	1.5%		69,475	Women from the general female population, reporting ever having had a clinical diagnosis of a genital STIs, ages 18-45.	Faber et al 2011
	UK	London, West Midlands and Local Authorities	2009–2011		Ranged 50+/100 000 to <0·01/100 000	3 221854	Data from the Genitourinary Medicine Clinic Activity Dataset (GUMCAD)***, women and man.	Mitchell et al 2013
Asia								
	India	Mysore	2005-2006	8.5%		898	Women were recruited from low-income peri-urban and rural neighbourhoods of Mysore, ages 15-30.	Madhivanan et al 2009
	South Korea	Cheonan	2006-2012	7.8%		1,523	Female patients attending STD clinics in the Dankook University Hospital for STI screening, ages 10–81.	Kim 2013
	South Korea	Daegu	2013	4%		201	Male patients from a primary care urology clinic (Top Urology Clinic).	Seo et al 2014
Oceania								
	Australia	South-East Queensland and indigenous rural regions	2004-2011	Prevalence were 2.1-fold higher in women than in men (1.5% vs 0.7%); Indigenous patients accounted for 48% of positive cases		44 464	Women and man from rural and urban Australian populations.	Bygott and Robson 2013
	Australia	New South Wales	2009-2010	8.4%		506	Women from rural and remote western New South Wales 23 years median age.	Ryder et al 2012
	Australia	Central and Northern Australia	2009-2011	17.6%	19.8/100 person-years for women and 3.6/100person-years for man.	17 849	Women and man from remote Australian Aboriginal populations .	Silver et al 2014

*NHANES—National Health and Nutrition Examination Survey

**CHOICE is an observational cohort study of 9,256 women residing in the St. Louis area.

***National STI surveillance and reporting system in England

Data for the US was the most robust, as many articles analyzed samples collected for National Health and Nutrition Examination Survey (NHANES), STD Surveillance Network (SSuN) and CHOICE data, which collects information from a population representative of the US. Prevalence estimates for the America region ranged from 14.4% for Atlanta to 0.85% for Grenada.[10,21] Data from Brazil, the second country with highest number of studies, ranged from 12.7% for ages 18-78 yr to 2.6% for ages 25-45 yr among women attending in healthcare units.[19,18] However, data from Brazil was the less robust due to the limitations in sample selection, since it is not representative of the Brazilian general population. The same limitation study was found for Grenada and Peru, where the data evaluated were restricted to the local population instead of being considered at national level. [21, 20] Reported prevalence estimates in Europe, among four Nordic countries,[29] tended to be lower than those reported for the America, Africa, Australia, and Asian regions (Table 1). Prevalence estimates for the Asian ranged from 8.5% for Mysore, India[24] among women to 4.0% for males living in Daegu, South Korea.[23] Data from Africa were the less robust; only one study has prevalence estimates for trichomonosis, 6.5% among women living in Durban, South Africa.[30] In addition, the available data from Africa in scientific literature were focused mainly on HIV-infected population, and this population was excluded from this study.

Oceania studies focused mainly on Australian aboriginal and communities living in rural areas. The prevalence ranged from 17.6% for aboriginal populations[27] to 8.4% for rural population, [26] indicating that in some communities trichomonosis causes a significant burden. For example, in Australia, the *T. vaginalis* infection estimates were higher in women than in men; [25] and the indigenous patients were positive cases in 48% of the studied population.[25]

Four studies reported prevalence of *T. vaginalis* among male patients, among these two articles reported data in the US, [11, 12] one article reported data in the UK,[28] and one article reported data in the South Korea.[23] The prevalence estimates among male patients ranged from 10% [11] to 4.0%, [23] and data among women ranged from 14.4%[10] to 0.85%.[21] An Australian study has shown that the prevalence was 2.1 times higher in women than in men; however, the limitations on the number of studies with male samples restricted the comparison of data (Table 1).

Risk factors associated with *T. vaginalis* infection were showed in Figure 3; we classify the risk factors in three categories: human behavior, other disease and demographic aspects. Thirteen (57%) of the twenty three studies included in this review provided risk factors for trichomonosis.[8,10,11,12, 13, 14, 16, 17, 18, 20, 24,28,29] Skin color was the most commonly cited risk factor for trichomonosis acquisition (Figure 3).

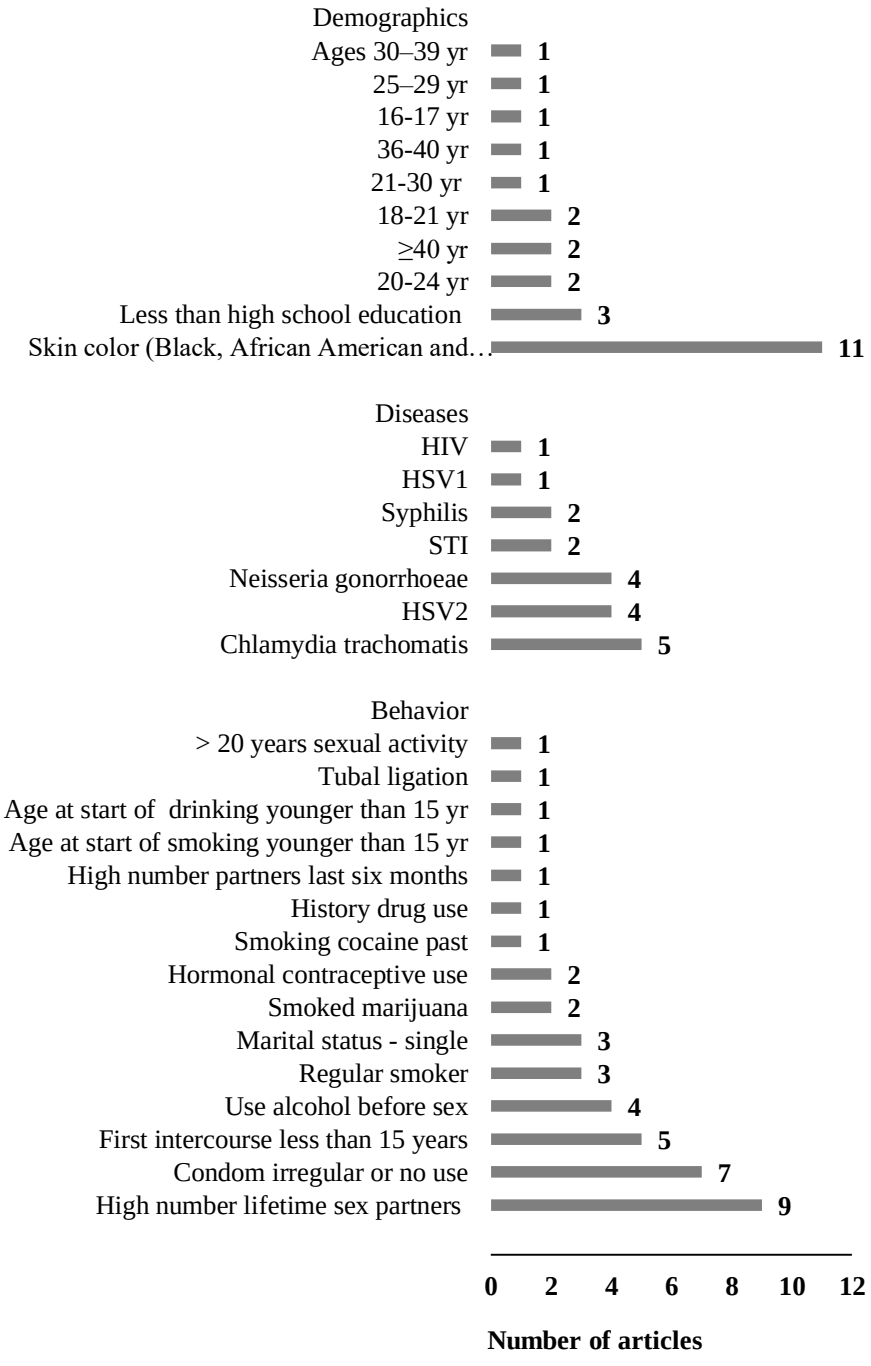

Figure 3. Articles citing risk factors for *T. vaginalis* infection.

Eleven studies cited Black, African American or Indigenous as a risk factor associated with *T. vaginalis*. Allsworth and colleagues[8] found the odds ratio of Non-Hispanic Black being infected compared to Non-Hispanic-Whites to be 12.3 (6.21-24.2) in the US.[8] In England, for both men and women, the odds of *T. vaginalis* infection were higher in Black Caribbean (women 4.23, 95% CI 3.98–4.50); (men 8.00, 95% CI 6.48–9.87) and Black 'other ethnic groups' (women 4.13, 95% CI 3.80–4.49); (men 5.75, 95% CI 4.22–7.83). Consequently, some ethnic groups were significantly more likely to be diagnosed with *T. vaginalis* than those who were White.[28] Other study also showed that some ethic groups were most probable to be infected with *T. vaginalis,* for example, data from Australia estimate that trichomonosis was highest among Aboriginal women than women living in remote areas.[27]

Considering demographic factors the low-level education was the second risk factor more associated with *T. vaginalis* infection.[11,8,20] Moreover, as observed in Figure 3, there is no peak of trichomonosis cases in certain age groups, the age of the population studied ranged from 16 to ≥ 40 yr.

The main diversity of risk factor cited for trichomonosis acquisition was among human behavior, which included the high number of sexual partners, lack of condom use, age of first sexual intercourse, use of alcohol, smoking cigarette or marijuana and other attitude, as shown in Figure 3.[8,10,11,12, 13, 14, 16, 17, 18, 20, 24,28,29]

The association between *T. vaginalis* and chlamydia, gonorrhea, syphilis, and herpes simplex virus-type 2 (HSV-2) have been described; and the most commonly cited diseases associated with trichomonosis in this review were *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and HSV2 infections (Figure 3). Allsworth and colleagues in 2009[8] using NHANES data in the US concluded that all sexually transmitted infections (STI) examined - chlamydia, HSV-1, HSV-2, syphilis and HIV- were more common among women with trichomonosis.[8]

Discussion

The findings in this systematic review indicated that prevalence estimative of trichomonosis is high in all five-world regions, in general population, since it frequently exceeding five percent. In the America region, especially in the US, it was found the

highest number of studies, highlighting two studies revealing prevalence of 10 percent or more for the US.[10,11] Consequently, data obtained in this review suggested that the overall trichomonosis burden is better known in the US. However, it has been reported that *T. vaginalis* has received little attention in the US and consequently the role of trichomonosis as important STI in US has probably been underestimated[6]. Recently the US Centers for Disease Control and Prevention (CDC) announced an initiative to prioritize five neglected parasitic infections, including trichomonosis,[5] making clear that the total *T. vaginalis* infection prevalence is still unknown in US. In addition, our data showed that, although the highest number of studies were found in the US, these data were collected in different cities, with heterogeneous population and, in different periods, and as a result, it is no possible to define whether the *T. vaginalis* prevalence is increasing. However, when we compare two large US studies, one covering the women population aging 15 to 49 yr throughout the country between 2001 and 2004[8] and the other considering women from 21 of 50 US states, ages 18-89 yr,[14] it is possible to conclude that the trichomonosis prevalence is increasing. Therefore, these results reaffirm that trichomonosis need greater attention of public health policies in US. Other interesting information is that data suggest that prevalence is lowest among teenager than women belonging to other age groups.[8,14]

Data from Brazil was the second in articles number, and the populations studied among all three studies were comparable when the age groups were analyzed. However, the Brazilian country region evaluated and time point were not comparable among these studies; consequently it was not possible to concluded whether the prevalence in Brazil was changing.[17,18,19]

Our results showed that among other four world-regions studied here, Africa, Asian, Europe, and Oceania there are more limited prevalence estimates data for trichomonosis than America region. Taking in account Africa and Asian regions, the studies included in the analysis did not represent all populations from these regions; therefore the overall burden of disease in Africa and Asian is still unidentified. [22, 23,24,30] The same limitation was found for Australian data, although a more representative population was studied, the Aboriginal Indigenous communities living in rural areas, than Africa and Asian.[25,27] In Europe, only one report established the prevalence

estimates of *T. vaginalis* infection in Nordic countries;[29] consequently, the number for this disease in a representative part of population is misjudged. Finally, hardly any new data about the *T. vaginalis* incidence were found in this review.

Trichomonosis, to the best of our knowledge, is not currently a reportable disease, nor is a nationally notifiable condition in any of the regions studied. Furthermore, the CDC Guidelines recommended that only women with symptoms of vaginal discharge should be tested for *T. vaginalis* infection and the screening of asymptomatic persons includes only routine annual testing among HIV-infected women and currently do not include specific recommendations for *T. vaginalis* screening among the general population or among other high-risk groups.[15] Consequently, these public health policies applied to trichomonosis may underestimate the prevalence numbers and incidence of this disease in the world, and this fact certainly explains the limited number of studies found in Africa, Asian, Europe, and Oceania. In addition, Meites and collegues[15] in the US representative population data found prevalence estimates of 6.5% of *T. vaginalis* positive cases among asymptomatic women. Therefore, due to the high number of asymptomatic patients with trichomonosis, the enhanced efforts such as screening for *T. vaginalis* among other population other than HIV-infected could lead to increased awareness about this infection and to treatment and reduction of transmission.

Limitations of this study include restrictions used in our search methodology, in particular the restricted inclusion of articles published within the past six years. An additional limitation was that most studies with quality to be included in this review belong to the US, so the prevalence and incidence data from worldwide is unknown. Another limitation was the highest number of studies in high-risk population, as sex-workers or HIV-positive people, as well as in pregnant women found during the systematic review. These studies were excluded, because the number of trichomonosis cases could be overestimated or underestimated when these populations are included.

Conclusion

Our overall purpose was to assess the available literature on the current burden of trichomonosis in five world-regions and to discern the amount of evidence available to inform current or future public health measures to control and prevent trichomonosis.

Our findings highlight the ongoing need for better data, especially because prevalence estimates are strong only in the US and incidence estimates are far from optimal in all world-region. Due the paucity of trichomonosis estimates, we are still in the nascent stages of understanding the full extent of trichomonosis in the world. Thus, efforts are necessary to better define the overall burden of disease, for example research to elucidate the trichomonosis prevalence and incidence numbers. Robust epidemiological studies of prevalence and incidence showing the disease ongoing are needed to guide priorities and define public health policies. In addition, there is the necessity to develop more widely accessible diagnostic tests and studies to re-evaluate and improve T. vaginalis screening guidelines.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary material

S1 Protocol search

S2 PRISMA checklist

S3 PRISMA flowchart

S1 Protocol search

Full description of protocol to study search and criteria of inclusion/exclusion used.

Search protocol

a) Search

The Pubmed search limits: English articles and articles publish before 01.01.2008 and 01.01.2015.

a.1) Terms that will include:

First step:

i) “trichomonosis prevalence”; ii) “trichomonas incidence”; ii) “trichomonosis prevalence and Americas”; iv) “trichomonosis prevalence and South-East” v) “trichomonosis prevalence and Asian”; vi) “trichomonosis prevalence and European”; vii) “trichomonosis prevalence and Eastern Mediterraneam”; viii) “trichomonosis prevalence and Pacific”

Second step:

i) “trichomonosis incidence and Americas”; ii) “trichomonosis incidence and South-East” iii) “trichomonosis incidence and Asian”; iv) “trichomonosis incidence and European”; v) “trichomonosis incidence and Eastern Mediterraneam”; vi) “trichomonosis incidence and Pacific”

Third step:

i) “*Trichomonas* vaginitis”; ii) “*Trichomonas* infection”; iii) “*Trichomonas* Urethrites”

B) Analyses of search results

b.1) Title selection for abstract review

i) Title report “prevalence or incidence of trichomonosis or *Trichomonas vaginalis*” ii) “prevalence or incidence of sexually transmitted disease or sexually transmitted infection”; iii) frequency or occurrence of sexually transmitted infection”; iv) “epidemiological study”.

b.2) Criteria for exclusion of abstract review

i) duplicate results; ii) Studies about different disease or other *Trichomonas* disease topic; ii) articles about *Trichomonas* in animals; iii) Studies in specific population i.e HIV patients, sex workers, pregnant women or other population.

b.4) Criterion of inclusion that will used in abstract review for study selection:

i) original prevalence data/estimate for trichomonosis; ii) original incidence or outbreak data; iii) report of individual cases.

b.4) Criterion for study inclusion in full-text review analyses

i) appropriate population selection; ii) appropriate methodological approach; iii) appropriate statistical analysis; iv) outcome description.

S2 PRISMA checklist

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	

Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	
DISCUSSION			

Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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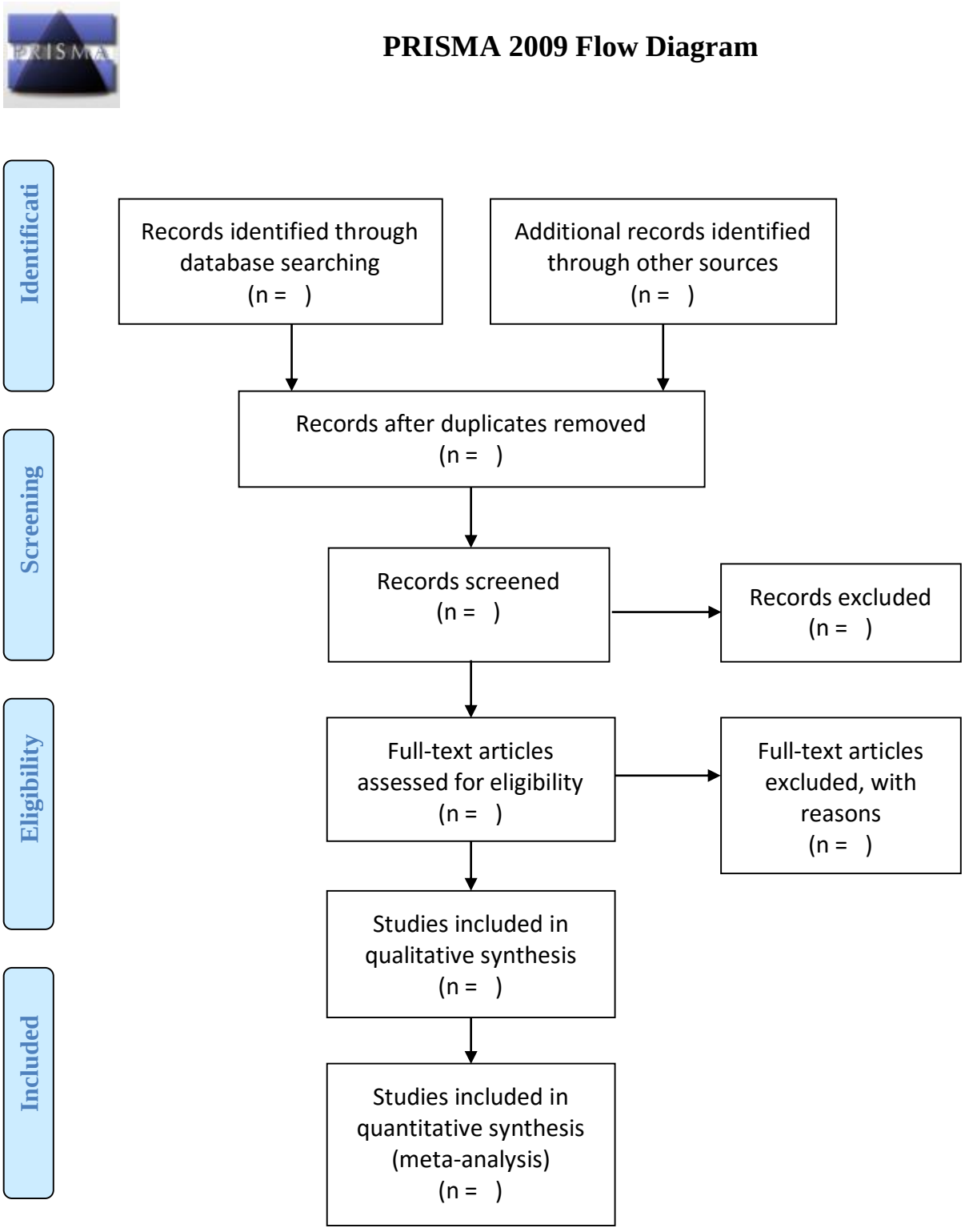
S3 PRISMA flowchart



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II.1.2 Artigo 2 Optimal Reference Genes for Gene Expression Normalization in *Trichomonas vaginalis.* Odelta dos Santos, Graziela de Vargas Rigo, Amanda Piccoli Frasson, Alexandre José Macedo and Tiana Tasca.

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RESEARCH ARTICLE

Optimal Reference Genes for Gene Expression Normalization in *Trichomonas vaginalis*

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Abstract

Trichomonas vaginalis is the etiologic agent of trichomonosis, the most common non-viral sexually transmitted disease worldwide. This infection is associated with several health consequences, including cervical and prostate cancers and HIV acquisition. Gene expression analysis has been facilitated because of available genome sequences and large-scale transcriptomes in *T. vaginalis*, particularly using quantitative real-time polymerase chain reaction (qRT-PCR), one of the most used methods for molecular studies. Reference genes for normalization are crucial to ensure the accuracy of this method. However, to the best of our knowledge, a systematic validation of reference genes has not been performed for *T. vaginalis*. In this study, the transcripts of nine candidate reference genes were quantified using qRT-PCR under different cultivation conditions, and the stability of these genes was compared using the geNorm and NormFinder algorithms. The most stable reference genes were *α-tubulin*, *actin* and *DNATopII*, and, conversely, the widely used *T. vaginalis* reference genes *GAPDH* and *β-tubulin* were less stable. The *PFOR* gene was used to validate the reliability of the use of these candidate reference genes. As expected, the *PFOR* gene was upregulated when the trophozoites were cultivated with ferrous ammonium sulfate when the *DNATopII*, *α-tubulin* and *actin* genes were used as normalizing gene. By contrast, the *PFOR* gene was downregulated when the *GAPDH* gene was used as an internal control, leading to misinterpretation of the data. These results provide an important starting point for reference gene selection and gene expression analysis with qRT-PCR studies of *T. vaginalis*.

Introduction

The most common non-viral sexually transmitted disease (STD) worldwide is trichomonosis that is caused by *Trichomonas vaginalis*, which is a flagellate parasitic protozoan, with an incidence estimated of 276.4 million new cases each year [1]. Recently, *T. vaginalis* was classified among five parasitic infection neglected in the United States of America [2]. Serious health consequences have been associated with trichomonosis such as infertility, predisposition to cervical

design, data collection and analysis, decision to publish, or preparation of the manuscript.

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cancer, pelvic inflammatory disease and adverse pregnancy outcomes like low birth weight babies and preterm birth [3,4]. In addition, aggressive prostate cancers have been associated with trichomonosis [5]. Other important aspect about the trichomonosis is a positive association between *T. vaginalis* infection and human immunodeficiency virus (HIV) transmission [6]. The complete genome from *T. vaginalis* was published in 2007 [7], consequently this could allow greatly progress in pathogenicity studies, as it has occurred with other organisms when the complete genomes and transcriptomes sequences were available [8,9]. For these approaches, the measurement of gene expression using quantitative real-time transcriptase reverse PCR (qRT-PCR) is chosen because it is rapid, sensitive and precise particularly to detect a few copies from mRNA [8, 10–12]. However, data analysis could have errors due to technical and experimental variability [13]. Therefore, to avoid the effect of these factors, the use of housekeeping genes is necessary [13, 14]. The low or absent variability in its expression level is expected for one reference gene when exposed to different types of experimental treatments [13, 15, 16]. However, frequently used housekeeping genes such as *glyceraldehyde-3-phosphate* (*GAPDH*), *elongation factor* and *β-actin*, have displayed unstable expression levels under different experimental studies, therefore demonstrating the importance of establishing a set of optimal reference genes, before starting a gene expression analysis [13,17,18]. Nevertheless, there are no reported studies of the validation of reference genes in *T. vaginalis*, and genes such as *α-* or *β-tubulin* are frequently used as housekeeping genes for normalization in *T. vaginalis* gene expression analyses [19,20]. In this study, nine candidate reference genes previously used in studies with *T. vaginalis* or other eukaryotes, were selected and their mRNA levels were measured in trichomonads under nutrient-deficient conditions and high iron conditions by qRT-PCR. Next, two algorithms NormFinder and geNorm were used to classify the suitable reference genes for the normalization of gene expression in *T. vaginalis* [15, 21]. To confirm the consistency of the selected reference genes, pyruvate:ferredoxin oxidoreductase (*PFOR*) was used as the target gene. *PFOR* is a hydrogenosomal enzyme that has homology with the 120 kDa surface glycoprotein (AP120) involved in the *T. vaginalis* cytoadherence process, and it is up-regulated in the presence of iron [22]. Therefore, the three most stable and one unstable candidate reference genes were selected for testing for normalization of the relative expression of *PFOR* in trichomonads cultivated under high iron concentrations. The results obtained here represent important information for reference gene selection to use in gene expression studies in *T. vaginalis*.

Results

Selection of candidate RT-qPCR reference genes for *Trichomonas vaginalis*

To identify a set of potential reference genes, a thorough review of the scientific literature with a focus on genes traditionally used as normalizers for gene expression in *T. vaginalis* studies was performed. Of five reference genes often used for expression assays: *β-tubulin* [19,22,23,24,25,26,27,28,29,30,31,32], *α-tubulin* [33,34,35,36,37,38], *60S rRNA* [39,40,41], *GAPDH* [38,42], and *coronin* [8], the three most used genes for normalization were selected, *β-tubulin*, *α-tubulin*, and *coronin*. An additional six candidate genes were included because of their use as the best reference genes for other organisms, and a total of nine candidate genes were selected, *actin*, *F-actin* (*β* and *α*), *tubulin* (*α*, *β* and *γ*), *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, *elongation factor (Efa)*, and *DNA topoisomerase II (DNATopII)* (Table 1). The nucleotide sequences of all candidate reference genes were obtained from the *T. vaginalis* genome project database (TrichDB http://trichdb.org/trichdb/), where more than one sequence was found. To select only one nucleotide sequence for each gene, the following approach was used: all sequences obtained with a score equal to 100 in the TrichDB search were aligned

Table 1. Gene ID, gene symbol, primer sequence and amplicon length of the selected reference genes.

Gene ID	Gene Symbol	Primer sequence (5' to 3')	Amplicon length (bp)
TVAG_534990	*Actin*	F: TCACAGCTCTTGCTCCACCA	175
		R: AAGCACTTGCGGTGAACGAT	
TVAG_212270	*F-actin α*	F: ATCGACGAAGGCATCAAAGC	103
		R: TACGAGCTTCCTCGCAAAGG	
TVAG_271840	*F-actin β*	F: TCTTCGGATGCGGTGTTTTC	178
		R: CCGATTCCAACGTCAAGCTC	
TVAG_206890	*α-tubulin*	F: TGCCCAACAGGCTTCAAGAT	101
		R: TTAGCGAGCATGCAGACAGC	
TVAG_073800	*β-tubulin*	F: TCCGTGGCCGTATGTCATCT	169
		R: GCTGTTGTGTTGCCGATGAA	
TVAG_109820	*γ-tubulin*	F: TGCCGATGCTCTTGAAGGAT	173
		R: TGTATGGGGCAACAACGACA	
TVAG_03880	*DNATopII*	F: ATCGGTGTCGGTTGGTCAAG	171
		R: TGGCTGTTTGACACCGTCTTT	
TVAG_067400	*Efa*	F: CACAACAACAGGCCACCTCA	136
		R: TTCAGCCTTGAGGGAGTCCA	
TVAG_475220	*GAPDH*	F: GCCGCAAGCTCTATCCAAAG	196
		R: CGGCCACCGATTGACTTAAC	

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using the ClustalW2 –Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and the phylogenetic analysis was performed using MEGA (Molecular Evolutionary Genetics Analysis software) (S1 Fig). Next, only one nucleotide sequence for each gene was selected from the branch subtypes with a greater number of individuals. The primer pairs specificities were verified with a BLAST (Basic Local Alignment Search Tool; at http://blast.ncbi.nlm.nih.gov/) search and the primer pairs designed were used only if they did not amplify human or other *Trichomonas* species sequences.

PCR Amplification Specificity and PCR efficiency

The specific melting temperature, corresponding to a single peak was found for all nine candidate reference genes (S2A Fig and Table 2) and a single band for each product was visualized in the agarose gel electrophoresis (S2B Fig), indicating the specificity of all primer pairs used for

Table 2. Results from standard curves of the selected candidate references genes: slopes, amplification efficiency (E), annealing temperature (Ta), melting temperature (Tm) and primer concentration.

Gene Symbol	Slope	E (%)	R^2	Ta (°C)	Tm (°C)	Primer concentration (μM)
Actin	-3.527	92	0.990	64	86.3	0.1
F-actin α	-3.318	100	0.986	64	81.5	0.1
F-actin β	-3.551	91	0.978	63	83.3	0.1
α-tubulin	-3.472	94	0.997	64	86.3	0.2
β-tubulin	-3.452	95	0.991	64	84.7	0.1
γ-tubulin	-2.787	110	0.994	64	80.3	0.2
DNATopII	-3.329	90	0.993	64	81.8	0.1
Efa	-3.504	99	0.996	64	84.8	0.2
GAPDH	-3.316	100	0.992	64	84.8	0.2

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qRT-PCR. The DNA calibration curves of ten-fold dilution series were used to calculate the regression coefficient (R^2) for each candidate reference gene, thus the primer efficiency was evaluated. The PCR efficiency range from 90% for *DNATopII β* to 110% for *γ-tubulin* is shown in Table 2. No primer dimers or unexpected amplicons were observed from non-specific amplification, and no signals were detected in the negative controls.

Expression levels of the selected candidate reference genes

To recognize the ideal reference genes for normalization of the gene expression profiles in *T. vaginalis*, the raw Ct values were used to measure the expression stabilities of the nine candidate genes. All data were examined under the following five subsets: (1) *T. vaginalis* grown in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated bovine serum (HIBS) (2); HIBS restriction (1.0%) (3); maltose restriction (273 μM); (4) HIBS and maltose restriction; and (5) TYM supplemented with 200 μM ferrous ammonium sulfate.

The expression levels of the nine reference genes for all sets of samples are presented in Fig 1 using the raw Ct values. Diverse levels of mRNA copy were observed for these genes, with the Ct values ranging from 8 cycles in *Efa* gene to 25 cycles for the *F-actin α* gene. A constant expression level was not found for none of the tested reference gene in all conditions tested. The dispersion of the Ct values was the lowest for the *Efa*, *α-tubulin*, *actin*, and *DNATopII* genes, indicating the lowest gene expression variations, whereas the *γ-tubulin* and *β-tubulin* genes showed the highest variability in the CT value, and consequently, the highest gene expression variations. Five candidate reference genes had average Ct values below 15 cycles, including *Efa*, *GAPDH*, *α-tubulin*, *actin*, and *γ-tubulin*, indicating higher expression levels. By contrast, *F-actin α*, *DNATopII*, *F-actin β*, and *β-tubulin* had average Ct values above 15, indicating that these genes produced fewer transcripts. Consequently, the *Efa* gene presented the highest abundant expression level, whereas the *F-actin α* gene had the lowest level.

The coefficient of variation (CV%) values were calculated for each candidate reference gene after grouping the samples into the following three different sets: (i) total samples (all growth conditions); (ii) all samples subjected to nutrient restriction; and (iii) samples cultivated with

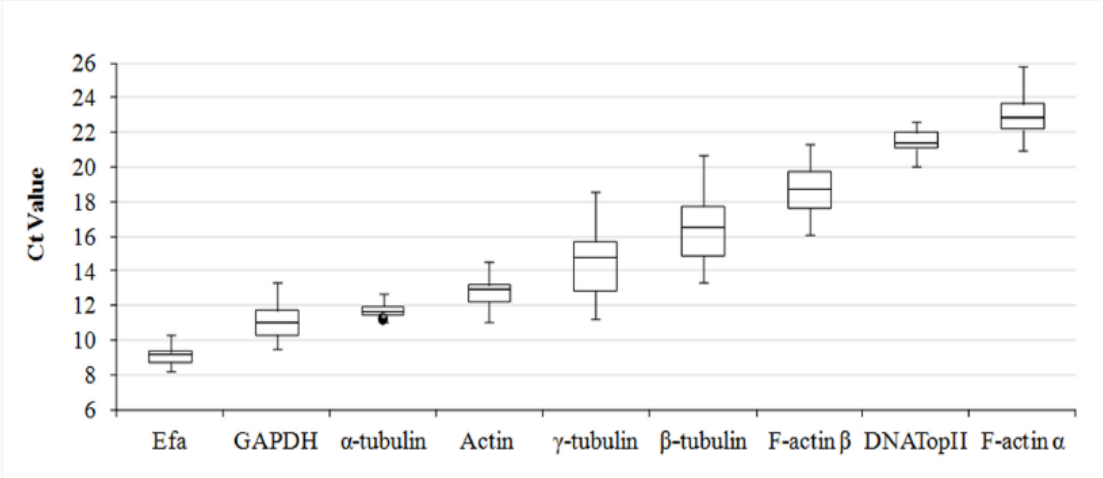

Fig 1. Raw Ct values from qRT-PCR of candidate reference genes under all cultivation conditions. Box shows the 25/75 percentiles and the *whiskers* indicate the maximum and minimum values. The median is presented by the line and outliers are exhibited by dots.

doi:10.1371/journal.pone.0138331.g001

Table 3. The coefficient of variation (CV %) of candidate reference genes from *T. vaginalis* under three distinct sets.

Gene	Total samples CV %	Gene	All nutrient restriction CV %	Gene	Ferrous ammonium sulfate CV %
DNATopII	2.9	*DNATopII*	2.42	*Actin*	2.5
F-actin α	4.4	*α-tubulin*	4.5	*F-actin α*	3.8
α-tubulin	4.7	*F-actin α*	4.9	*F-actin β*	4.4
Efa	5.0	*Actin*	5.2	*α-tubulin*	4.7
Actin	6.2	*Efa*	5.7	*DNATopII*	7.6
F-actin β	6.3	*F-actin β*	6.4	*Efa*	11.9
β-tubulin	10.0	*β-tubulin*	9.9	*β-tubulin*	39.2
GAPDH	10.1	*GAPDH*	10.3	*γ-tubulin*	39.6
γ-tubulin	13.4	*γ-tubulin*	13.2	*GAPDH*	40.3

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ferrous ammonium sulfate. The CV was calculated as CV = σ/μ, where σ is the standard deviation of the Ct values of a candidate reference gene and μ is the Ct value mean for the same gene. These values were also evaluated to verify the expression stability for each candidate gene. CV values lower than 4.0% were obtained for *DNATopII* when all samples were analyzed and when the samples from the nutrient restriction were grouped into one set (Table 3). Of the other potential reference genes, CVs lower than 4.0% were obtained for *actin* and *F-actin α* when the parasites were cultivated with ferrous ammonium sulfate. Conversely, *β-tubulin*, *GAPDH*, and *γ-tubulin* presented high CV values under the three conditions sets, with CV values ranging from 9.9% to 40.3% (Table 3).

Expression stability of the candidate reference genes

Two different statistical algorithms NormFinder and geNorm were used to rank the candidate reference genes according to their expression stability [43].

geNorm analysis. The geNorm was used to calculate the expression stability value (*M*) for each candidate reference gene and the pairwise variation (*V*) of a certain gene compared with others. To do the analysis using the geNorm software the raw Ct values were grouped into the following six experimental datasets: (i) total samples; (ii) all samples under nutrient restriction; (iii) samples under HIBS restriction; (iv) samples under maltose restriction; (v) samples under HIBS and maltose restriction; and (vi) samples supplemented with 200 μM ferrous ammonium sulfate.

The average expression stability (*M* value) of the tested genes is based on the average pairwise expression ratio. A low *M* value indicates more stable gene expression, whereas the highest *M* value denotes the least stable reference gene. All candidate reference genes in all six subsets tested showed *M* values lower than the geNorm threshold of 1.5, revealing stability (Fig 2). In the entire set of 15 samples, *DNATopII* (0.225) and *α-tubulin* (0.239) had the lowest *M* values, followed by *F-actin α* and *actin*. Conversely, the *M* value of *GAPDH* was the highest (0.685), suggesting that *DNATopII* and *α-tubulin* present the most stable expression and that *GAPDH* is variably expressed (Fig 2A). The results remained similar in the high-iron experimental subset (Fig 2F), with the lowest *M* values for *α-tubulin* (0.051), *DNATopII* (0.06), and *actin* (0.068), and the highest *M* values for *β-tubulin* (0.505). By contrast, *β-tubulin* (0.182) and *actin* (0.17) were more stable when the complete data sets of the nutrient restriction were grouped together (Fig 2B), and *GAPDH* and *Efa* had highest *M* values, with the lowest expression stability. The *α-tubulin* (0.114) and *actin* (0.119) genes were ranked as the most stable under HIBS restriction (Fig 2C), whereas *F-actin α* (0.038) and *DNATopII* (0.034) were ranked as the most stable under maltose restriction (Fig 2D). For these two last experimental subsets, the unstable

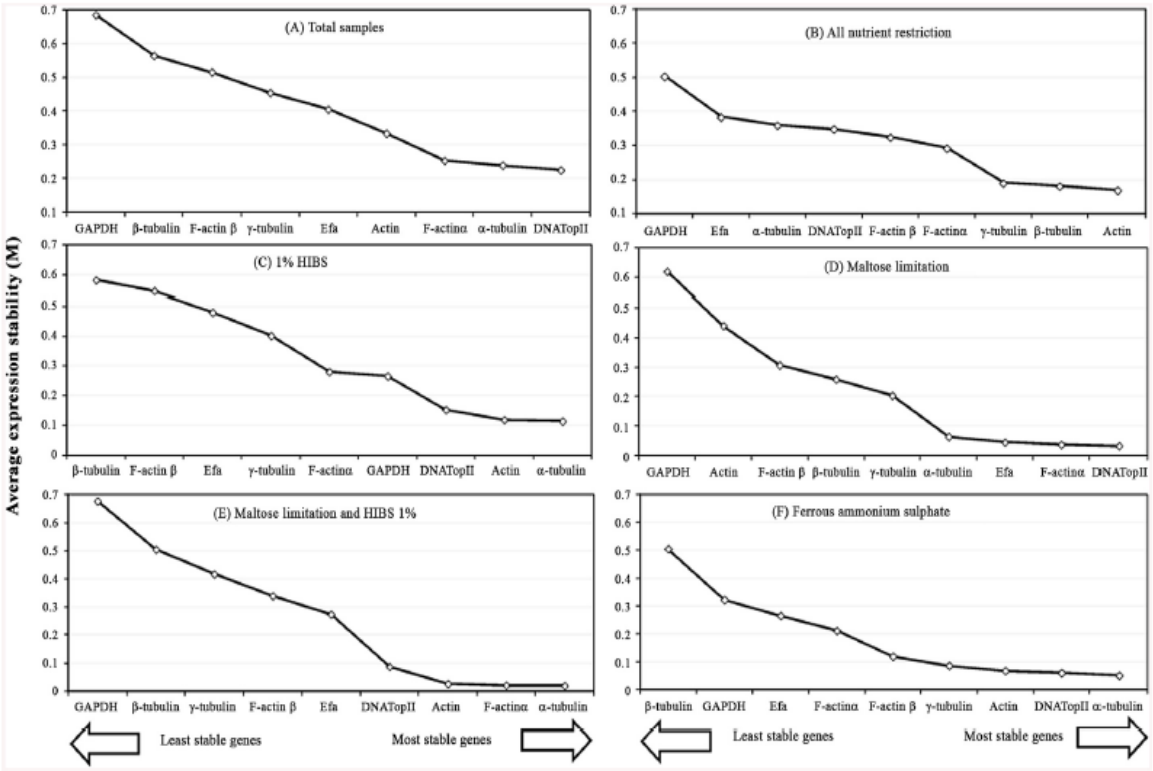

Fig 2. Expression stability values (*M*) and ranking of the candidate reference genes based on geNorm calculation. (a) total samples; **(b)** samples under all nutrient restriction; **(c)** samples with 1% HIBS; **(d)** maltose restriction; **(e)** HIBS and maltose restriction; **(f)** samples supplemented with 200 μM ferrous ammonium sulfate. The *M* value and ranking were calculated through a pairwise comparison and stepwise exclusion of the lowest stable gene. Low *M* values correspond to high expression stability.

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genes were *β-tubulin* plus *F-actin α* and *GAPDH* plus *actin*, respectively (Fig 2C and 2D). When there was an association between both nutrient restriction conditions (HIBS and maltose), *F-actin α* (0.022) and *α-tubulin* (0.02) had the most stable expression, in contrast to *GAPDH*, which was variably expressed (Fig 2E).

The pairwise variations (V*n*/V*n* + 1) were also calculated with geNorm between two sequential ranked normalization genes to determine the minimum number of internal controls needed for an accurate normalization. Analysis of the pairwise variation in all datasets revealed that the optimal number of reference genes in these experimental situations were two, and geNorm *V* was < 0.15 for all subsets compared to a normalization factor based on the 2 or 3 most stable targets (Fig 3).

NormFinder analysis. To evaluate the stability of the nine candidate reference genes using the NormFinder algorithm, the samples were grouped into the following two sets: (i) control samples and (ii) all nutrient restriction samples. The expression stability evaluated by the NormFinder program is shown in Table 4. The reference genes with the lowest average expression stability were more stably expressed reference genes. Based on our results, the three most stable reference genes were *DNATopII*, *α-tubulin* and *actin*, and the best combination of two genes were *DNATopII* and *α-tubulin*. When the samples under nutrient restriction were grouped in the same subset, the *α-tubulin*, *actin*, *DNATopII* genes also exhibited the lowest average expression stability; however, the best combination of two genes was *α-tubulin* and *actin* (Table 4).

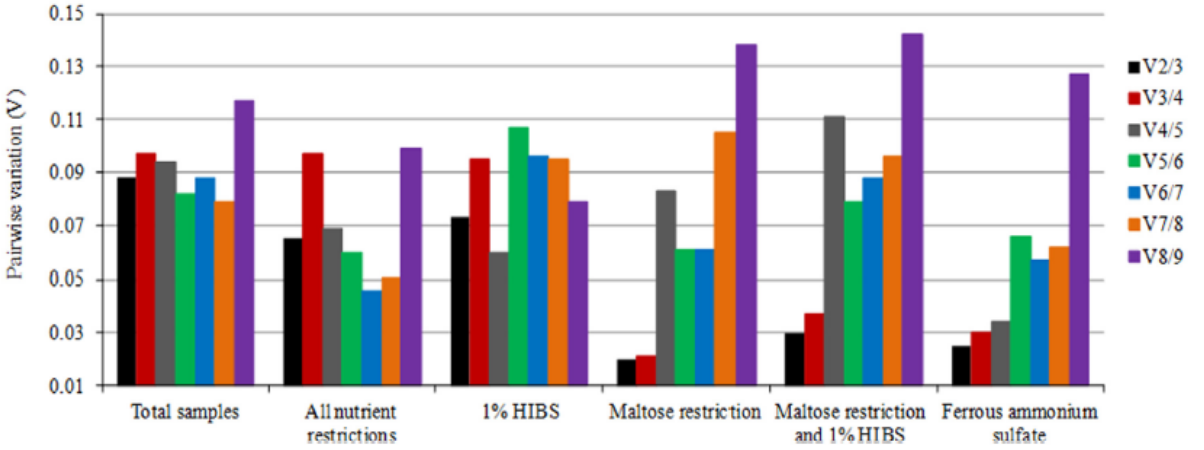

Fig 3. Pairwise variation (*V*) generated by geNorm to identify the optimal number of internal controls. *V* values less than 0.15 indicate that no additional genes are needed to calculated a reliable normalization factor.

doi:10.1371/journal.pone.0138331.g003

Validation of the selected reference genes in parasites supplemented with ferrous ammonium sulfate

In order to ensure the reliability of the selected reference gene candidates, we analyzed the expression patterns of one iron up-regulated gene (*PFOR*) in trophozoites previously treated with increasing ferrous ammonium sulfate concentrations (100, 200, and 300 μM) at four time points (1, 6, 12, and 24h). For these experiments three *T. vaginalis* isolates, one ATCC (30238) and two fresh clinical isolates (TV-LACM6 and TV-LACH4) were used. The *actin* and *DNA-TopII* genes were selected because of their optimal performance in the geNorm and NormFinder analyses. By contrast, the unstable gene *GAPDH* was also evaluated.

As presented in Figs 4, 5 and 6 *PFOR* gene had higher expression pattern among all three *T. vaginalis* isolates tested for 1, 6, 12 and 24 h of incubation with ferrous ammonium sulfate when the most stable candidate reference genes (*actin* and *DNATopII*) were used for normalization.

Table 4. Ranking of candidate reference genes in order of expression stability as calculated by NormFinder.

Rank	Total samples			Rank	All nutrient restriction		
	Gene	Stability value	$BC_2G^{(+)}$		Gene	Stability value	$BC_2G^{(+)}$
1	*DNATopII*$^{(+)}$	0.024	0.047	1	*α-tubulin*$^{(+)(a)}$	0.021	0.016
2	*α-tubulin*$^{(+)}$	0.081	0.047	2	*Actin*$^{(+)}$	0.021	0.016
3	*Actin*	0.089	-	3	*DNATopII*	0.026	-
4	*F-actin α*	0.272	-	4	*F-actin α*	0.065	-
5	*Efa*	0.289	-	5	*Efa*	0.316	-
6	*F-actin β*	0.307	-	6	*F-actin β*	0.403	-
7	*γ-tubulin*	0.362	-	7	*γ-tubulin*	0.449	-
8	*GAPDH*	0.599	-	8	*GAPDH*	0.605	-
9	*β-tubulin*	1.132	-	9	*β-tubulin*	1.348	-

$^{(+)}$ Best combination of two genes and stability value for best combination of two genes

$^{(a)}$ Best gene

doi:10.1371/journal.pone.0138331.t004

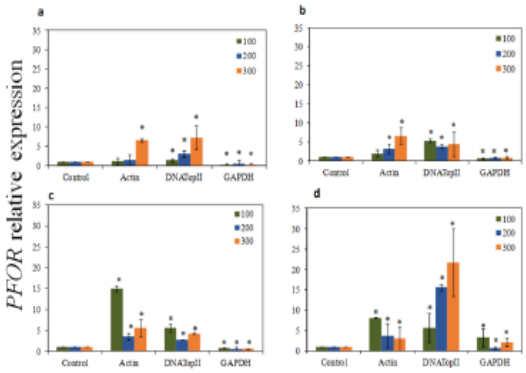

Fig 4. The quantification of *pyruvate-ferredoxin oxidoreductase* (*PFOR*) gene expression in *T. vaginalis* ATCC 30238 isolate. The relative expression of *PFOR* gene in *T. vaginalis* under ferrous ammonium sulfate (high-iron condition 100, 200, and 300 μM) using *GAPDH*, and *DNATopII* as internal controls, after 1, 6, 12, and 24h of cultivation. (a) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron after 1 hour of cultivation; (b) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 6 hours of cultivation; (c) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 12 hours of cultivation; (d) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 24 hours of cultivation. The relative expression levels are depicted as the mean ± SD, calculated from three biological replicate. The relative change in gene expression was analysed using the $2^{-\Delta\Delta Ct}$ method. Statistically significant expression changes were calculated using one-way ANOVA and the level of significance was also determined by the Bonferroni method comparing all groups versus the control. Statistically significance ($P < 0.001$) changes in relative expression are represented with an asterisk.

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Using the other stable reference gene *α-tubulin*, our qRT-PCR analysis also revealed that the *PFOR* transcripts were upregulated on parasites treated with iron at 24h exposition ($P > 0.05$) (S3 Fig). In contrast, when *GAPDH* was used as reference gene, the relative expression of the *PFOR* target varied greatly, showing lower expression level in all experimental conditions tested ($P > 0.05$) than results obtained from normalization with *actin*, *DNATopII*, and *α-tubulin*. Moreover, in Figs 4, 5 and 6 it is also observed that the increase in the *PFOR* gene expression accompanied the increase in iron concentrations. Similarly, the raise in *PFOR* expression was time-dependent, with strongest expression in 24 h. These expression patterns were found among all three *T. vaginalis* isolates tested when were used the stable genes as normalizing. However, normalization using *GAPDH* gene resulted in a strong bias, due to significant decrease of the *PFOR* expression despite increase in both iron concentration and time (Figs 4, 5 and 6).

Discussion

An ideal reference gene should have stable expression level in all tissues, as well as in all organisms culture conditions [13, 15, 16]. However, currently some studies showed that the expression of traditionally used housekeeping genes may diverge when tested under different experimental treatments [17, 18, 43]. Regarding this concern, a set of potential nine reference genes were selected in this study to compare their expression stabilities under different experimental conditions to establish the optimal reference genes for the normalization of qRT-PCR analyses in *T. vaginalis*.

The nine-candidate reference genes evaluated here showed a relatively wide range of transcript levels when the raw Ct data was analyzed, and these findings were observed in other studies of gene validation [44]. The *Efa*, *GAPDH*, *α-tubulin* and *actin* genes had low data dispersion suggesting they were most stable genes. These results were consistent with previous studies that evaluated *Efa*, *GAPDH*, *actin*, and *tubulin* as potential reference genes and

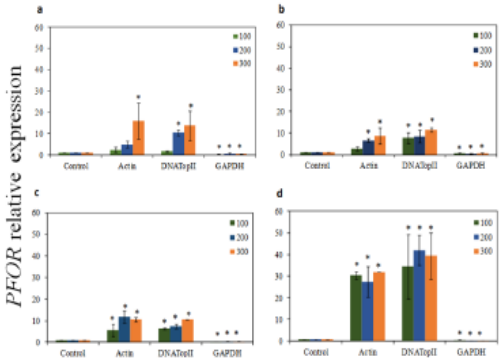

Fig 5. The quantification of *pyruvate-ferredoxin oxidoreductase* (*PFOR*) gene expression in *T. vaginalis* TV-LACM6 isolate. The relative expression of *PFOR* gene in *T. vaginalis* under ferrous ammonium sulfate (high-iron condition 100, 200, and 300 μM) using *GAPDH*, and *DNATopII* as internal controls, after 1, 6, 12, and 24h of cultivation. (a) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron after 1 hour of cultivation; (b) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 6 hours of cultivation; (c) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 12 hours of cultivation; (d) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 24 hours of cultivation. The relative expression levels are depicted as the mean ± SD, calculated from three biological replicate. The relative change in gene expression was analysed using the $2^{-\Delta\Delta Ct}$ method. Statistically significant expression changes were calculated using one-way ANOVA and the level of significance was also determined by the Bonferroni method comparing all groups versus the control. Statistically significance ($P < 0.001$) changes in relative expression are represented with an asterisk.

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confirmed that these genes were appropriate as a reference panel for normalizing gene expression data [45, 46]. When average of raw Ct values were analyzed, to estimate expression levels for each candidate reference gene, we found higher expression levels to *Efa*, *GAPDH*, *α-tubulin*, *actin*, and *γ-tubulin*, on the other hand, the *actin*, *GAPDH* and *tubulin* genes had fewer

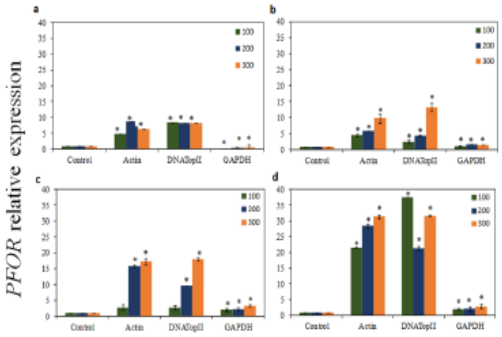

Fig 6. The quantification of *pyruvate-ferredoxin oxidoreductase* (*PFOR*) gene expression in *T. vaginalis* TV-LACH4 isolate. The relative expression of *PFOR* gene in *T. vaginalis* under ferrous ammonium sulfate (high-iron condition 100, 200, and 300 μM) using *GAPDH*, and *DNATopII* as internal controls, after 1, 6, 12, and 24h of cultivation. (a) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron after 1 hour of cultivation; (b) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 6 hours of cultivation; (c) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 12 hours of cultivation; (d) The relative expression of *PFOR* gene in *T. vaginalis* under high-iron condition after 24 hours of cultivation. The relative expression levels are depicted as the mean ± SD, calculated from three biological replicate. The relative change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Statistically significant expression changes were calculated using one-way ANOVA and the level of significance was also determined by the Bonferroni method comparing all groups versus the control. Statistically significance ($P < 0.001$) changes in relative expression are represented with an asterisk.

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transcripts when evaluated in *Litsea cubeba* [45]. However, there are not previous studies of the validation of reference genes in *T. vaginalis* limiting data comparison. Therefore, our contribution is valuable for future studies on *T. vaginalis* gene expression analysis.

For an ideal reference gene, a constant Ct value is expected and consequently, low CVs, which ideally should be less than 4.0% [47]. In this study, only three genes had CVs values lower than 4.0% (Table 2). However, the direct comparison of the raw Ct values should be avoided, because they did not result in a precise estimation of the expression stability since the data obtained from the raw Ct values falsely represents the variation in gene expression [48]. Therefore, more powerful methods should be used to evaluate the expression variation of candidate reference genes. In the present study, we used two algorithms, geNorm analysis, which can screen stably expressed genes and determine the optimal number of internal controls, and NormFinder analysis, which calculates the stability value for each gene and ranks the genes.

In geNorm analysis, the nine candidate reference genes in all six sample subsets showed lower *M* values than the geNorm threshold (> 1.5), suggesting that all nine genes tested here are stable. A previous report also described low *M* values for distinct experimental subsets studied, displaying high expression stability [14,46]. However, when the data obtained here were analyzed for each subset, it was possible to determine that there was a difference in the *M* values for all potential reference genes in each sample subsets; therefore, it was possible to classify the genes as most or least stable. *GAPDH* was the most unstable gene in four experimental subsets, as previously shown for chickpea [49]. This is in contrast to a previous report in human keratinocyte cell lines, in which *GAPDH* was the most stable gene [50]. The *β-tubulin* gene, the most used housekeeping gene for both qRT-PCR and RT-PCR in *T. vaginalis* studies, was also one of the most unstable genes of the five sample subsets. These results confirm that the selection of reference genes for normalization based on the traditional use as housekeeping genes is an inappropriate approach [17, 18]. *DNATopII*, *α-tubulin*, *F-actin α* and *actin* were the most stable of the nine genes tested and of the six experimental subsets, although some changes in the ranking in each subset occurred. Hence, our findings suggest that the most adequate reference genes were *α-tubulin* and *actin*. These observations confirm the fact that there is no universal reference gene and point to the need of specific optimization of potential reference genes before starting any experimental condition [13].

The optimal number of internal reference genes for normalization was also determined with the geNorm software, which generates the pairwise variation *V* value. The results of all samples subsets showed that the use of two of the most stably expressed reference genes as internal control genes was sufficient because the $V_{2/3}$ was less than 0.15. Therefore, the inclusion of the other genes did not have any effect on the normalization factor.

The ranking of the potential reference genes generated by NormFinder were slightly different from geNorm. The *DNATopII*, *α-tubulin* and *actin* genes were ranked as the most stable in all samples, and the best combination of two genes was *DNATopII* and *α-tubulin*. These two genes were also the most stable genes when all samples were analyzed with the geNorm software. When all nutrient restriction subsets were analyzed with NormFinder, *α-tubulin*, *actin* and *DNATopII* were ranked as the most stable genes, and the best combination was *α-tubulin* and *actin;* however, in the geNorm analyses, the two most stable genes were *actin* and *β-tubulin*.

The most unstable genes were *γ-tubulin*, *GAPDH* and *β-tubulin* for the NormFinder analyses, and *Efa* and *GAPDH* for geNorm. The variation obtained between these two software programs has been described in previous reports on reference genes validation [44,45, 46]. Based on the three analytical tools used, the CV values, NormFinder and geNorm, the most unstable genes were *β-tubulin*, *GAPDH* and *γ-tubulin*. However, both the *β-tubulin* and *GAPDH* genes have been widely used as housekeeping genes in *T. vaginalis*, and surprisingly in our study, these two genes were ranked as the most unstable reference genes in different samples subsets.

Moreover, when the raw Ct data were evaluated, the *GAPDH* gene had a minor dispersion of data, suggesting stability. This result confirms that in qRT-PCR analyses, the statistical data should be converted to the linear form by the 2^{-CT} calculation and should not be presented as the raw Ct values [48]. Therefore, the use of both *β-tubulin* and *GAPDH* as reference genes should be avoided in qRT-PCR analysis in *T. vaginalis*. The combination of *α-tubulin* and *actin* should be used as a reference gene in most of sample subsets because *F-actin α* and *DNA-TopII* had fewer transcripts and were unsuitable for the normalization of target genes with higher expression levels. Consequently, our results confirm the previously published data, which showed that the stability of gene expression is based on the experimental condition and not only on the species tested [14].

We evaluated the expression levels of the *PFOR* gene in samples previously treated with ferrous ammonium sulfate using *DNATopII*, *α-tubulin*, *actin*, and *GAPDH* as the reference genes. We confirmed that the relative expression profile of *PFOR*, an iron-up-regulated gene, were consistent when using *α-tubulin*, *actin* and *DNATopII*, and the combination of two genes as reference genes. Moreover, a slight difference in *PFOR* expression was observed when *DNATopII*, *α-tubulin*, *actin* were used, although there was no significant difference in *PFOR* expression. In contrast, the normalized expression level showed a reduction in *PFOR* expression when *GAPDH* was used as an internal control, indicating that it is the most unstable gene when parasites are cultivated with ferrous ammonium sulfate.

To reaffirm our results we expanded the number of *T. vaginalis* isolates using two fresh clinical isolates. The TV-LACM6 isolate presents remarkable characteristics: high ability to adhere to plastic and to human vaginal epithelial cells, high cytolysis (unpublished data) and the isolate harbors *Mycoplasma hominis* and one *T. vaginalis* viruses (TVV) specie (TVV 1) [51]. The isolate TV-LACH4 harbors four distinct TVVs species (TVV 1, 2, 3, and 4) as previously shown by our group [51]. Thus, we evaluated the *PFOR* expression level among all three isolates (ATCC 30238, TV-LACM6, and TV-LACH4) under different ferrous ammonium sulfate concentration in different time points. So, we confirm that the relative expression profile of *PFOR* were in good consistency with increasing ferrous ammonium sulfate concentrations and cultivation times, when *DNATopII* and *actin* were used as reference genes. However, the normalized expression level of the target showed a reduction in expression when using *GAPDH* as internal control, independently of ferrous ammonium sulfate concentration used or time. Consequently, these results are inconsistent with the *PFOR* expression profile, which is a known iron up-regulated gene. Therefore, our findings reinforce that the use of unsuitable internal control may result in data misinterpretation.

Thus, these results reaffirm the reliable use of *DNATopII*, *α-tubulin* and *actin* in combination as a reference in *T. vaginalis* studies, since the *PFOR* expression profile were consistence with the increase of ferrous ammonium sulfate concentration in different *T. vaginalis* clinical isolates with different genetic and virulence characteristics. Finally, our study confirms the fact that there is no universal reference gene and warns the need of specific optimization of potential reference genes before starting any experimental condition [13,14].

Conclusion

In the present study, we validated nine candidate reference genes by subjecting the parasites to distinct growth conditions, including HIBS and maltose restriction, as well as supplementation with ferrous ammonium sulfate. The *α-tubulin*, *actin* and *DNATopII* genes exhibited the most stable expression in the majority of samples. Conversely, *GAPDH* and *β-tubulin*, the most used genes in *T. vaginalis* studies, were the most unstable. In addition, we suggest that the use of two genes, *α-tubulin* and *actin*, should be sufficient to provide reliable results. To the best of our

knowledge, this study is the first systematic exploration of *T. vaginalis* to identify optimal reference genes for qRT-PCR normalization under different culture conditions. This is valuable for future research on *T. vaginalis* gene expression studies.

Material and Methods

Identification of normalizer genes in previous *T. vaginalis* studies

A review on the literature was performed to identify the most common genes used as normalizers in *T. vaginalis* studies. A search of the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) using the keywords "*Trichomonas vaginalis* and qRT-PCR" and "*Trichomonas vaginalis* and RT-PCR" was performed to determine the usual normalizer genes used in quantitative or not quantitative RT-PCR.

Trichomonas vaginalis culture and experimental conditions

Trichomonas vaginalis trophozoites, isolate 30238 from the American Type Culture Collection, were cultured axenically *in vitro* in a trypticase-yeast extract maltose (TYM) medium (pH 6.0) supplemented with 10% heat-inactivated bovine serum (HIBS [v/v]) and incubated at 37°C (±0.5) [52]. Organisms exhibiting motility and normal morphology during the logarithmic growth phase were harvested, centrifuged, washed three times with phosphate-buffered saline 1X (PBS) (pH 7.0) and resuspended in fresh TYM medium for subsequent experiments.

Different experimental treatments were used to evaluate the performance of the selected candidate reference genes under four nutritional conditions. For these treatments, $1.0x10^5$ trophozoites/mL were incubated in TYM containing 1.0% HIBS (serum restriction), 273 μM maltose (maltose restriction), 1.0% HIBS and 273 μM maltose (serum plus maltose restriction) and 200 μM ferrous ammonium sulfate (high-iron concentration), for 24 hours at 37°C. The control group represents parasites cultured with TYM containing 27.3 mM maltose and supplemented with 10% HIBS.

To evaluate *PFOR* gene expression in parasites supplemented with ferrous ammonium sulfate, the *T. vaginalis* fresh clinical isolates TV-LACM6 and TV-LACH4 were included in this study. These isolates were obtained at Laboratório de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFRGS, Brazil, and were registered and stored by cryopreservation at -80C° in the *T. vaginalis* isolates bank of our research team (this survey was submitted and approved by the UFRGS Ethical Committee, number 18923). The fresh clinical isolates were grown under the same conditions as described above. All treatments were performed on three different days.

Primer designs

The Primer3 designing software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used to design primer pairs using the following criteria: product size between 100 and 200 bp, Tm of approximately 60°C, GC content of 40–60% and primers length of 18–22 bp. The generated primer pair for each gene was then aligned against the *T. vaginalis* genome to confirm its specificity *in silico*. The primer pairs were also evaluated for primer dimer formation using multiple primer analyzers (http://www.thermoscientificbio.com/webtools/multipleprimer/). The forward and reverse primers were intentionally targeted to the adjoining exons, which were separated by an intron. The gene ID, primer sequence and gene symbol are shown in Table 1 for all nucleotide sequences assessed here.

PCR efficiency and specificity

A 10-fold serial dilution consisting of five samples starting from 100 ng genomic DNA was used to construct standard curves to determine the PCR amplification efficiencies (E) for each

candidate reference gene. The DNA was extracted from ~4.0 x10^6 trophozoites/mL using the AxyPrep Multisource Genomic DNA Miniprep Kit (BioScience, Inc) according to the manufacturer's recommendations. The PCR amplification specificity for each candidate reference gene was assessed by melting curve analyses and agarose gel electrophoresis. Three technical replicates from three biological replicates were analyzed.

RNA extraction

The total RNA was extracted from ~4.0 x10^6 trophozoites/mL using TriZol™ following the manufacturer's instructions and was stored at -80°C until use. The integrity of the RNA samples was determined by 2.0% agarose gel electrophoresis and well-defined bands that confirmed the absence of nucleic acid degradation. The quantity and purity of the RNA were determined using spectrophotometric method, and only the high-quality samples, in which the A_{260}/A_{280} was 1.8 and A_{260}/A_{230} was 2.0, were used for subsequent qRT-PCR analyses. The total RNA samples were pretreated with DNAse, to ensure that there was no contamination with genomic DNA in the qRT-PCR analysis.

Real-time PCR analyses and quantitative reverse transcriptase PCR (qRT-PCR) amplifications

To determine the primer standard curve, real-time PCR reactions were performed in 0.1-mL microtubes using the Qiagen real-time PCR system, Rotor-Gene Q and Rotor-Gene™ SYBR™ Green RT-PCR kit (Qiagen™). Each PCR reaction contained 6 μL of 2x Rotor-Gene SYBR Green PCR Master Mix, 100 nM or 200 nM (Table 1) of each primer and 2 μL of genomic DNA template in a total volume of 12 μL. The annealing temperatures and primer concentration were selected for the highest amplification, best product specificity and no primer dimer amplification based on the melting curve analyses. The cycling conditions were as follows: initial enzyme activation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 s and annealing and extension at 63°C or 64°C (Table 1) for 30 s, with fluorescence data collection recording 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. No DNA template controls were also included for each primer pair as a negative control. For quantitative reverse transcription, 100 ng of RNA and 0.125 μL Rotor-Gene RT Mix were added to each reaction. The qRT-PCR cycling was an initial step at 55°C for 10 min, followed by polymerase activation and PCR cycling as described above. Parallel reactions without both RNA template and transcriptase reverse enzyme were used as the negative control. Three biological replicate samples were analyzed in three technical replicates for each experimental condition.

Expression level of the selected candidate reference genes

The values of the cycle threshold (Ct) for each reaction were calculated by the Rotor-Gene Q series software 2.1.0 and these values were used to determine the average Ct for each sample. At least two of three technical replicates were considered, and any replicate showing non-specific products in the melting curve analyses was excluded from the average Ct calculation. The Ct averages from the technical replicates obtained from three biological replicates was used as the raw Ct data.

Data analyses

To determine the best reference genes among the different culture conditions, two independent statistical algorithms were used, geNorm and NormFinder [15,21]. For the geNorm

analysis, the raw data Ct values were entered into the geNorm of the qBase^{PLUS} V 2.4 software [53], and for the NormFinder, the raw data were transformed to the relative quantities using the delta-Ct method Q = $2^{-\Delta Ct}$ [48]. NormFinder was used to calculate the stability value of the reference genes based on their intra- and inter-expression variation, and those that exhibited lower average expression stability values were regarded as more stably expressed reference genes. The geNorm software was used to calculate the expression stability value (M) and the mean pairwise variation (V value, $V_{n/n+1}$) between all of the tested genes. The threshold of $V < 0.15$ was used in this study [15,21].

Validation of the selected reference genes

To validate the selected reference genes, the two most stable genes and the most unstable gene were used to analyze the relative expression levels of pyruvate-ferredoxin oxidoreductase (PFOR), which is an iron-up-regulated gene. For this assay, trichomonads from fresh clinical isolates, TV-LACM6 and TV-LACH4 were cultured in increasing iron concentrations (100, 200, and 300 μM) and the total RNA was extracted in different times: 1, 6, 12, and 24 hours. The total RNA was pre-treated with DNase I (Invitrogen^{TM}) following the manufacturer's instructions prior to the qRT-PCR. The *PFOR* primers were as follows: $PFOR_{AP120:}$ forward 5′ CTCGTTTGGGGTGCTACATT 3′ and reverse 5′ TCCTGATCCCAAACCTTGAG3′ (TVAG_198110; 239 bp). Three biological replicates were performed and the transcripts of the *PFOR* genes were quantified by qRT-PCR, and three technical replicates were performed for each sample. The relative change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method [48]. The *actin*, *α-tubulin* and *DNATopII* genes identified in this study as reference genes were used for normalization. The *GAPDH* gene was tested as the least stable reference gene. Statistically significant expression changes were calculated using one-way ANOVA. The level of significance was also determined by the Bonferroni method comparing all groups versus the control ($P < 0.001$).

Supporting Information

S1 Fig. Phylogenetic trees constructed from the candidate reference genes sequences. (A) *Actin*; (B) *F-Actin β*; (C) *α-tubulin*; (D) *β-Tubulin*; (E) *γ-tubulin*; (F) *GAPDH*; (*) the asterisks represent the sequences used in this study.
(TIF)

S2 Fig. Confirmation of primer specificity and amplicon size. (A) Melting curve of nine candidate reference genes. (B) Agarose gel (2.0%) showing the specific RT-qPCR product of the expected size for each gene. M represents a 2080 bp DNA marker.
(TIF)

S3 Fig. The quantification of *pyruvate-ferredoxin oxidoreductase* (*PFOR*) gene expression. The relative expression of *PFOR* gene in *T. vaginalis* under ferrous ammonium sulfate (high-iron condition 200 μM) using *GAPDH*, *α-tubulin*, *actin*, and *DNATopII* as internal controls, after 24h of cultivation. The relative expression levels are depicted as the mean ± SD, calculated from three biological replicate. The relative change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Statistically significant expression changes were calculated using one-way ANOVA and the level of significance was also determined by the Bonferroni method comparing all groups versus the control. Statistically significance ($P < 0.001$) changes in relative expression are represented with an asterisk.
(TIF)

Author Contributions

Conceived and designed the experiments: OS TT. Performed the experiments: OS GVR APF. Analyzed the data: OS. Contributed reagents/materials/analysis tools: AJM TT. Wrote the paper: OS APF AJM TT.

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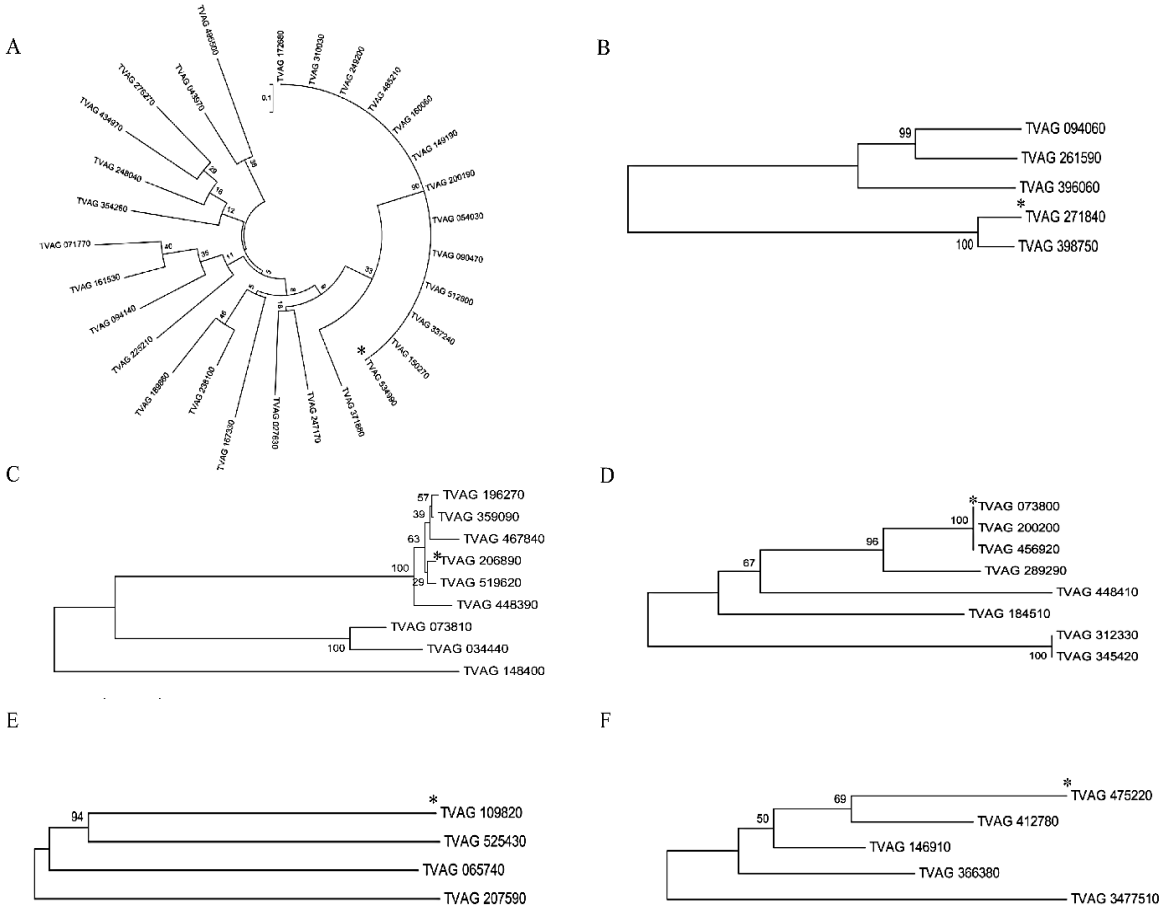


Figure S2

S1 Fig. Phylogenetic trees constructed from the candidate reference genes sequences. (A) *Actin*; (B) *F-Actin β*; (C) *α-tubulin*; (D) *β-Tubulin*; (E) *γ-tubulin*; (F) *GAPDH*; (*) the asterisks represent the sequences used in this study. doi:10.1371/journal.pone.0138331.s001

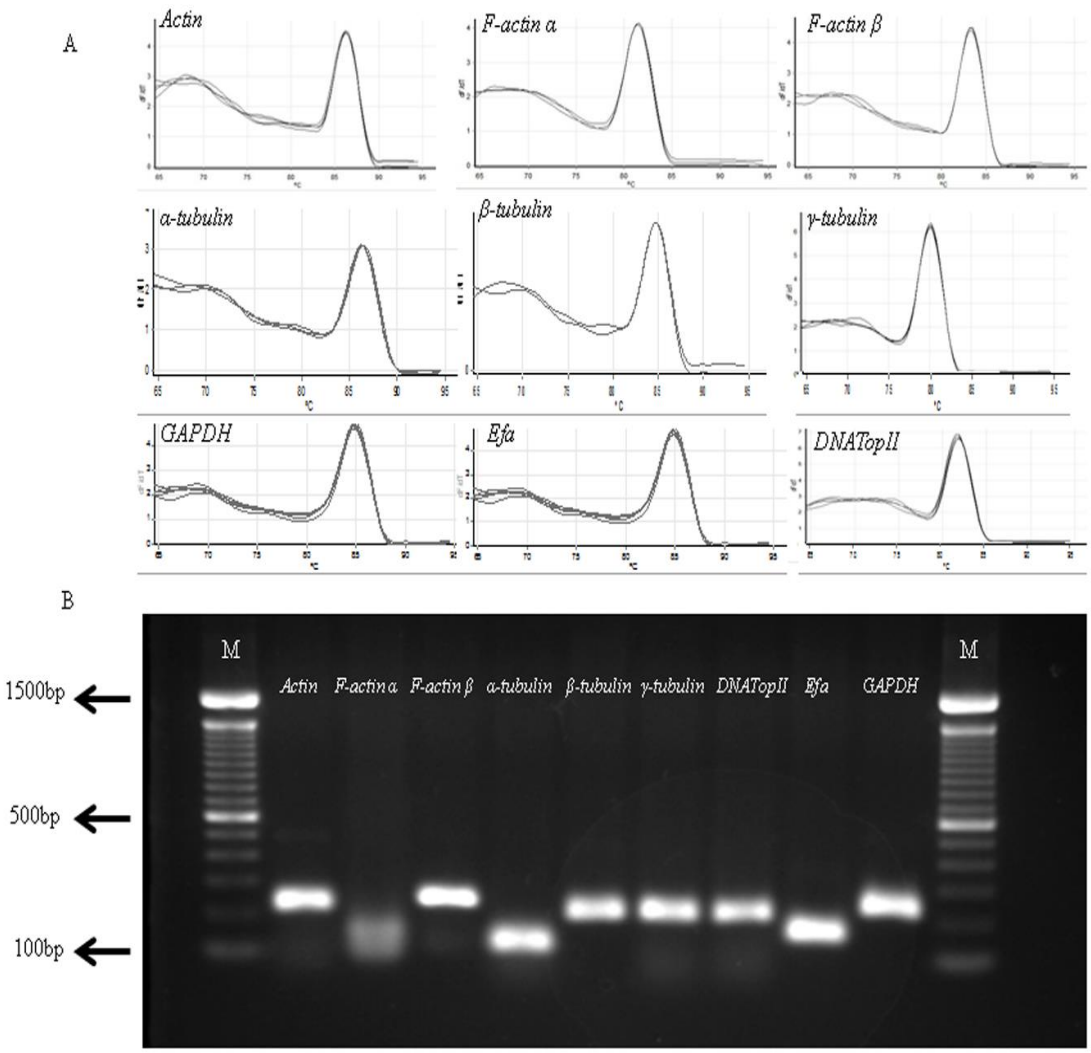
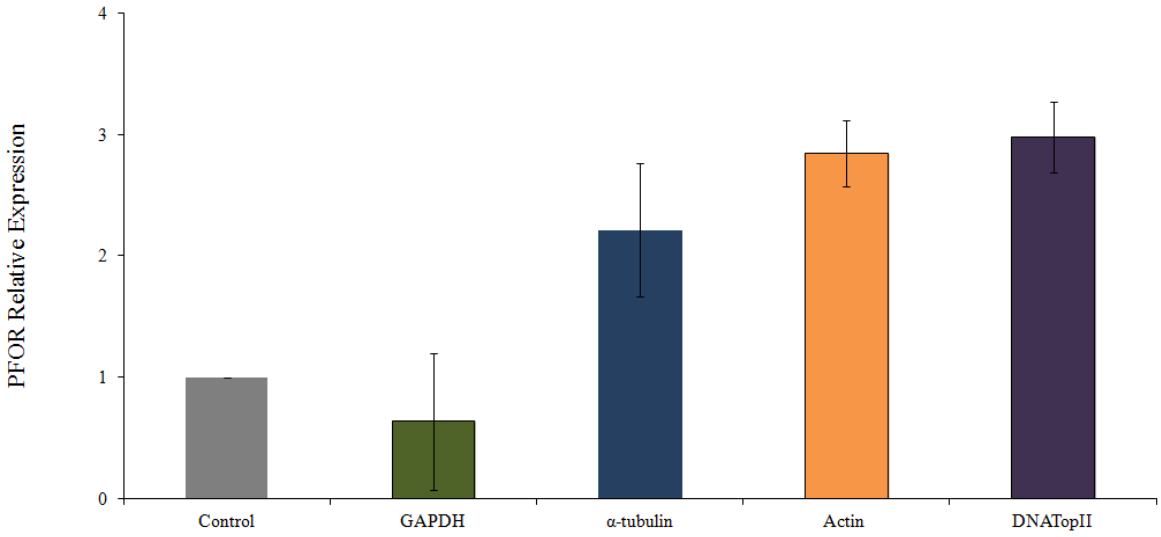


Figure S2.

S2 Fig. Confirmation of primer specificity and amplicon size.

(A) Melting curve of nine candidate reference genes. (B) Agarose gel (2.0%) showing the specific RT-qPCR product of the expected size for each gene. M represents a 2080 bp DNA marker.doi:10.1371/journal.pone.0138331.s002



S3 Fig. The quantification of pyruvate-ferredoxin oxidoreductase (PFOR) gene expression.

The relative expression of *PFOR* gene in *T. vaginalis* under ferrous ammonium sulfate (high-iron condition 200 μM) using *GAPDH*, *α-tubulin*, *actin*, and *DNATopII* as internal controls, after 24h of cultivation. The relative expression levels are depicted as the mean ± SD, calculated from three biological replicate. The relative change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Statistically significant expression changes were calculated using one-way ANOVA and the level of significance was also determined by the Bonferroni method comparing all groups versus the control. Statistically significance ($P < 0.001$) changes in relative expression are represented with an asterisk.doi:10.1371/journal.pone.0138331.s003

Author Contributions

Conceived and designed the experiments: OS TT. Performed the experiments: OS GVR APF. Analyzed the data: OS. Contributed reagents/materials/analysis tools: AJM TT. Wrote the paper: OS APF AJM TT.

II.1.3 Artigo 3. *Trichomonas vaginalis* clinical isolates: comparison among cytoadherence and adherence to plastic, intrauterine device, and vaginal ring. Odelta dos Santos, Graziela de Vargas Rigo, Alexandre José Macedo, and Tiana Tasca.

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Trichomonas vaginalis clinical isolates: comparison among cytoadherence and adherence to plastic, intrauterine device, and vaginal ring

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Abstract

The host parasitism by *Trichomonas vaginalis* is complex and in part is mediated by adherence to human vaginal epithelial cells (VECs). Five trichomonad surface proteins named adhesins and a glycoconjugate called lipophosphoglycan (LPG), play a role in the adherence of parasite to human cells. The adherence to plastic has been associated with virulence of *T. vaginalis* isolates, and to the best of our knowledge, no study evaluated the mechanism of adherence to plastic and epithelial cells simultaneously. Herein we investigate the adherence ability of *T. vaginalis* fresh clinical isolates to plastic, intrauterine device (IUD) and vaginal ring. The influence of LPG, cytoskeletal components of the parasite, surface molecules and the gene expression of four adhesins proteins in human cell attachment and in adherence to plastic, were also investigated. Of 32 *T. vaginalis* isolates studied here, 19 (59.37%) were able to adhere to polystyrene microplates, and five (15.6%) were strong adherent, fourteen (43.75%) were moderate adherent and thirteen (40.65%) were no or weak adherent. The *T. vaginalis* isolates TV-LACM6 and TV-LACM14, which were strong plastic-adherent, were also able to adhere to IUD and vaginal ring. Following chemical treatments, the *T. vaginalis* components, LPG, cytoskeletal proteins and other surface molecules were involved in both adherence to plastic and cytoadherence. The gene expression level from four adhesion proteins were highest in trophozoites adhered to CEVs than trophozoites adhered to abiotic surface. These results indicate that *T. vaginalis* presents multiple and

complex mechanisms for attachment to host cells and to abiotic surfaces, ensuring successful parasitism and consequent infection.

Keywords: *T. vaginalis,* adherence to abiotic surface, cytoadherence, intrauterine device, vaginal ring.

1. Introduction

Trichomonas vaginalis, a protozoan parasite, is the causative agent of trichomonosis, the most common non viral sexually transmitted disease (STD) in humans (WHO, 2012). In men, the infection is usually asymptomatic, although it may cause irritating urethritis or prostatitis, and recently it has been associated to aggressive prostate cancers (Sutcliffe et al, 2012). On the other hand, in women the disease is associated with a wide spectrum of clinical signals ranging from a relatively asymptomatic state to severe vaginitis with a foul-smelling discharge (Petrin et al 1998). Trichomonosis, in addition to being a cause of serious discomfort to women, also has been associated with adverse pregnancy outcome, manifested by preterm rupture of membranes, preterm delivery, low-birth-weight infants, infertility (COTH et al 1991; MINKOFF et al 1994; GIMENES et al 2014), cervical cancer (VIIKKI et al 2000), and increase in the transmission of human immunodeficiency virus. (VAN DER POL et al 2008).

Taking into account that *T. vaginalis* is an obligate extracellular mucosal pathogen, adherence to human epithelial cells is critical for parasite survival (PETRIN et al 1998). Adherence of the parasite to a variety of epithelia indicates that *T. vaginalis* has a promiscuous mechanism for attachment to host cells and/or the ability to use multiple adherence factors (OKUMURA, BAUM AND JOHNSON, 2008). Adherence of the parasite is thought to be mediated by five trichomonad surface proteins, reportedly AP23, AP33, AP51, AP65, and AP120, and the role of these proteins in cell adherence has been characterized. (ALDERETE and GARZA, 1985; ENGBRING and ALDERETE, 1998; ALDERETE et al 1995; MORENO-BRITO et al 2005). These surface proteins are the most extensively studied molecules in tricomonad cytoadherence. (BASTIDA-COURCUERA, 2005).

The ability of *T. vaginalis* to adhere to plastic surfaces in the presence of various agents and under different growth conditions was showed in wells of microtitre plates (GOLD and OFEK in 1992. SILVA-FILHO, ELIAS and SOUZA in 1987). Bastida-Courcuera et al (2005) showed that a glyconjugate called lipophosphoglycan (LPG), which is the main component from glycocalyx of T. vaginalis, plays a role in the adherence and cytotoxicity of parasites to human cells. In the same way, these authors showed that T. vaginalis LPG mutant parasites exhibited reduced adherence to plastic (BASTIDA-COURCUERA, 2005). The adherence to plastic has been associated with in vivo virulence of T. vaginalis isolates (GOLD, 1993), and to the best of our knowledge no study evaluated the mechanism of adhesion to plastic and epithelial cell together or the adherence ability of T. vaginalis to intrauterine device and vaginal ring. In addition, the role of adhesion proteins in adherence to plastic is unknown; furthermore, the LPG has been pointed as the mechanism preferred to plastic adhesion, or most studied (Bastida-Courcuera, 2005).

The intra-uterine device (IUD) is the second most popular contraceptive method used in world (WCU, 2011), because is the most cost-effective reversible contraception (TRUSSELL, 2012) and presents extremely low failure rates similar to those with female sterilization (STEINER, TRUSSELL and JOHNSOn 2007). However, some studies suggested that IUD use may increase the risk of STD acquisition. (CERUTI et al 1994; HAUKKAMAA et al 1986; ROY, 1991). The positive association between women using IUD and trichomonosis cases was described. (KAZEROONI and MOSALAEE, 2002). In the same way, Nasir et al (2005) found highest *T. vaginalis* prevalence rates in IUD users than no-users. The trichomonosis prevalence rate was 34% among women using IUD against only 5% among non-IUD users.

The use of vaginal rings (VR) has been preferred due to its efficacy and adherence to therapy while potentially decreasing toxic side effects when compared to daily pill oral administration (VALENTA, 2005); consequently this approach has become popular in contraception and in estrogen replacement therapy. (YOO and LEE, 2006). In addition, vaginal delivery of antiviral agents from VRs constitutes a potential route for human immunodeficiency virus (HIV) pre-exposure prophylaxis in women, particularly in the developing world. (NEL et al 2009; SAXENA et al 2009). Therefore, we attempt

to study if *T. vaginalis* was able to adhere to intrauterine devices and vaginal rings. (OKUMURA, BAUM and JOHNSON, 2008).

Considering the role of adherence to the epithelial cells and to abiotic surfaces for *T. vaginalis* pathogenesis, the aims of this study were: (i) to evaluate the ability of *T. vaginalis* isolates to adhere to plastic by testing a large collection of fresh clinical isolates; (ii) to determine whether T. vaginalis trophozoites have ability to adhere to IUD and vaginal rings; (iii) to determine the influence of parasite cytoskeletal components and surface molecules in the process of human cell attachment and adherence to plastic; (iv) to determine the relative adhesins expression in trophozoites attached to VECs and in the plastic surface.

2. Material and Methods

2.1 T. vaginalis cultivation and clinical isolates

A total of 30 fresh *T. vaginalis* isolates were obtained from urine samples. The parasites were harvested consecutively at Laboratório de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFRGS, Brazil, registered and stored by cryopreservation at -80Cº in the *T. vaginalis* isolates bank of our research team (this survey was submitted and approved by the UFRGS Ethical Committee, number 18923). Two isolates from the American Type Culture Collection (ATCC) were also used in this study, *T. vaginalis* 30236 and 30238. Trichomonads were cultured axenically *in vitro* in trypticase-yeast extract-maltose (TYM) medium (pH 6.0) supplemented with 10% heat-inactivated bovine serum (HIBS [v/v]), and incubated at 37°C (±0.5) (Diamond, 1957). Organisms exhibiting motility and normal morphology during the logarithmic growth phase were harvested, centrifuged, washed three times with phosphate-buffered saline (PBS-1x) (pH 7.0), and resuspended in new TYM medium.

2.2 Adherence to plastic in polystyrene microplate

In order to determinate the ability of T. vaginalis isolates to adhere to microplate polystyrene the methodology previously described by Bastida-Corcuera et al (2005) was employed. Briefly, trophozoites were cultured overnight in TYM medium and washed three times with warm 37°C (±0.5) HIBS-free TYM. Trophozoites were resuspended in warm TYM to 2.5 × 105 cells/mL and 2.0 mL of the suspension were incubated in triplicate in 24-well plates for 4 h at 37°C (±0.5). After incubation, wells were washed three times with HIBS-free warm TYM to remove unattached parasites. Wells were then incubated with crystal violet (0.13% crystal violet/5:2 ethanol-formaldehyde) for 5 min and washed three times with PBS-1x. Remaining dye was solubilized with 1% SDS in 50% ethanol and measured in a plate reader at 570 nm. All experiments described in this report were performed five times in triplicate.

2.3 Adherence to intrauterine device (IUD) and vaginal ring

To determine whether *T. vaginalis* was able to adhere to IUD and vaginal ring the following three *T. vaginalis* isolates were selected according to non-adherence to plastic, ATCC 30236, and strong adherence to plastic, TV-LACM6 and TV-LACM14. The threads from IUDs, that are the intrauterine-tails located in the vaginal cavity, were cut in 5 cm pieces, five of these pieces were placed into microtubes and *T. vaginalis* was added at a final concentration of 2.5×10^5 trophozoites/mL in 2.0 mL final volume of supplemented TYM medium and incubated at 37°C (±0.5) for 24h. After the incubation period, the thread pieces were transferred to new microtubes and washed gently three times with PBS-1x to exclude the detached trophozoites. The attached trophozoites were released from threads by trypsinisation and the number of organisms was counted using hemocytometer by trypan blue dye exclusion.

In order to determine the ability of *T. vaginalis* adherence to vaginal ring, one slice (3 cm) was placed in the bottom of 24 well microplates, and the vaginal ring slice was maintained in the bottom of the well by using a homemade device. The trophozoites concentration used as well as the experimental conditions were the same described above for the assays on IUD-threads adherence. Following the incubation, the TYM medium was aspirated and the wells were washed twice with warm PBS-1x to remove

the non-attached parasites. In sequence, the vaginal ring slices were placed in new microtubes and attached trophozoites were released by trypsinisation. The trophozoites adherence results were expressed in mm^2. Data were obtained by at least three independent experiments in triplicate.

2.4 Confocal microscopy

The confocal microscopy was used as a confirmation approach of adherence ability to intrauterine device and vaginal ring. Two strong adherent isolates were used: TV-LACM6 and TV-LACM14. The adherence assay to IUD-threads and vaginal ring was performed as previously described in materials and methods section, after that the attached trophozoites were fixed with 0.2 % glutaraldehyde plus PEM buffer for 15 minutes at room temperature. The devices were washed with PBS-1x and were labeled using 1 µM fluorescent probe taxoid FLUTAX-2 for 30 minutes at 37ºC and with 10µg/mL of DAPI for 15 minutes at room temperature. Images were obtained using an Olympus IX81 confocal microscope and UPLSAPO 60X WNA 1:20 objective and were overlaid using Olympus FV 1000 software.

2.5 Dip Coating

The threads from IUDs and vaginal ring pieces were covered with metronidazole before adhesion assay. These slices were incubated with metronidazole (100 mg/mL) at room temperature overnight. Slices were then washed in phosphate-buffered saline (PBS-1x) three times and dried in a laminar flow cabinet for 2h. After that, the adherence assay and confocal microscopy were performed as described in materials and methods section.

2.6 Adherence to human vaginal epithelial cells (HMVII) and analyses by flow cytometry

Human vaginal epithelial cells of the HMVII lineage (from the European Collection of Cell Cultures, ECACC) were grown on 24-well plates in Dulbecco's

Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg/mL penicillin-streptomycin. Cells were seeded on plates at 1.0×10^5 cells/well and were grown until 80-90% confluence for two days, prior to use in the adherence assay. The adherence to HMVII was determinate using two *T. vaginalis* isolates (TV-LACM6 and TV-LACM14), randomly selected among five isolates strong-adherent to plastic. After chemical treatment, the fluorescent CellTracker Orange CMTMR dye (Invitrogen) (0.5 µM) was used to stain the parasites and to discriminate them from HMVII cells as previously described by Brooks et al (2013).

The medium of confluent HMVII was aspirated and 250 uL of CellTracker-stained *T. vaginalis* was added at final concentration of 5×10^5 trophozoites/mL (BASTIDA-CORCUERA et al 2005; BROOKS et al 2013). The plates were incubated for 30 min at 37°C (±0.5) and 5% CO2. After incubation, the supernatants were aspirated and the wells were washed twice with warm PBS-1x to remove the non-attached parasites. The attached cells were released from wells by trypsinisation and quenched with addition of heat-inactivated bovine serum. After being released from the wells the parasites (500 uL total volume, trypsin plus bovine serum, 1:1, v/v), were analyzed by flow cytometry using BD FACSVerse (BD Biosciences) flow cytometer. Absolute counts were determined using the following equation: (number of events / sample volume acquired) x 0.5 (dilution factor) = absolute count in 10,000 events.

2.7 Chemical treatments of trichomonads for adherence to plastic and cytoadherence experiments

Two *T. vaginalis* fresh clinical isolates were used (TV-LACM6 and TV-LACM14) to evaluate the adherence factors of *T. vaginalis* in both abiotic and biotic surfaces. The trophozoites were chemically treated before plastic adherence and cytoadherence evaluations. Reagents that affect cytoskeletal functions in trichomonads were used: 0.2 µg/mL colchicine and 0.1 µg/mL cytochalasin B. Trichomonads were also treated with 10 mM sodium (meta) periodate used for the oxidation of surface carbohydrates, specially LPG. To evaluate the role of *T. vaginalis* surface proteins in the adherence and cytoadherence processes, trophozoites were treated with 1.0 mg/mL

trypsin. Finally, in order to have a control of adherence impairment that would not directly affect the surface, parasites were treated with 10 µg/mL metronidazole (MTZ), that certainly harms the trophozoites metabolism (Alderete and Garza, 2005).

For all above mentioned treatments the following methodology was used: the trophozoites were counted at 24 hours, harvested in log phase by centrifugation (4000 x g for 5 min), and washed three times with PBS-1x or HIBS-free TYM. The parasites were suspended in TYM or PBS-1x (trypsin and cytochalasin B) supplemented with drug, finally a suspension of 1.0×10^6 organisms per mL was pretreated for 30 min at 37°C (±0.5). After incubation, the trophozoites were washed three times with warm HIBS-free TYM, suspended in serum-TYM, counted, monitored for motility and viability by using trypan blue dye exclusion. After the chemicals treatments, the trophozoites were revaluated regarding the ability of plastic adherence and cytoadherence as previously described in material and methods.

For determination whether the reduction of adherence caused by chemical treatments could be reversible, an aliquot of 1mL of chemically treated trophozoites was re-inoculated into drug-free medium and after 24 hours at 37°C (±0.5) of incubation the trophozoites were counted and have their ability of adherence to plastic revaluated.

Statistically significant in both reduction of plastic adherence and cytoadherence by any of the above described treatments was calculated using one-way ANOVA; the level of significance was also determined by the Bonferroni method comparing all groups versus control ($P < 0.05$). All results were obtained by at least three independent experiments in triplicate.

2.8 Surface adhesins proteins gene expression in attached parasites

To evaluate the possible role of four adhesins in adherence to plastic and cytoadherence processes in *T. vaginalis* the mRNA levels of these proteins were evaluated in plastic and VECs attached trophozoites. For this, the total RNA was extracted from attached trophozoites and from parasites cultivated for 24 hours from the tubes that were used as control.

The total RNA was extracted using TriZol™ and treated with DNAse I (Sigma-Aldrich™) following the manufacturer's instructions and stored -80ºC until use. Five 10-fold series dilutions starting from 100 ng genomic DNA was used by constructing standard curves in order to determine PCR amplification efficiencies, all PCR efficiencies were greater than 95%. Quantitative Real Time PCR reactions (qRT-PCR) were carried out in 0.1 mL microtube using Qiagen's real-time PCR system - Rotor-Gene Q and Rotor-Gene™ SYBR™ Green RT-PCR kit (Qiagen™). Each PCR reaction contained 6.0 µL 2 x Rotor-Gene SYBR Green PCR Master Mix, 200 nM each primer, 0.125 µL Rotor-Gene RT Mix and 100 ng of RNA template in a total volume of 12 µL. The cycling conditions were the following: initial step at 55ºC for 10 min for reverse transcription and enzyme activation step at 95ºC for 10 min, followed by 35 cycles of denaturation at 95ºC for 15s and annealing and extension at 62 ºC for 30 s with fluorescence data collection recording in this step. Melting curve analyses was performed by raising the temperature at the end of each run in by 1ºC per 5s from 62 ºC to 95 ºC. No-DNA template controls were also included for each primer pair as negative control. Parallels reactions without RNA template and RNA template obtained only from VECs were runs as negative control. Three biological replicate samples were analyzed in three technical replicates to each experimental condition. The primers pair used in this study are described in Table 1.

Table 1. Primer sequences of adhesin proteins, TvNTPDases enzymes and reference genes.

GenBank ID	Primer name	Sequence (5'-3')	Amplicon length (bp)	Reference
U87093.1	TVap51	F-GCTTTCAGACCCAGTCGAAG	258	This study
		R-GCTTTCAGACCCAGTCGAAG		
U87096.1	TVap33.1	F-GCCAGTATGGCACAAAGGTT	222	This study
		R-GCTGTGGGATGTGTTCTGTG		
U18346.1	TVap65.1	F-GCTGAAGCTACACCACACGA	229	This study
		R-TGACGAGGGAAGCAAGAGAT		
AY652962.1	$AP120_{PFOR}$	F-CTCGTTTGGGGTGCTACATT	239	Santos et al, 2015
		R-TCCTGATCCCAAACCTTGAG		
TVAG_534990	Actin	F: TCACAGCTCTTGCTCCACCA	175	Santos et al, 2015
		R: AAGCACTTGCGGTGAACGAT		

The relative change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The levels of mRNA were quantified by qRT-PCR

analysis relative to *Actin* mRNA as internal controls as previously determined by our research team (Santos et al2015).

Statistical significance in the expression changes of adhesins between attached and unattached *T. vaginalis* in both processes of adherence to plastic and cythoaderence was calculated using one-way ANOVA; the level of significance was also determined by the Bonferroni method comparing all groups versus control ($P < 0.05$).

3. Results

3.1 Trichomonas vaginalis adherence to plastic in polystyrene microplates

In a previous study (Becker et al 2015), our group isolated thirty T. vaginalis from fresh clinical samples and they were evaluated in this study for the ability to adhere to polystyrene microplates. Two ATCC isolates (30236 and 30238) were included in this study, totalizing 32 *T. vaginalis* isolates. Of 32 isolates, 19 (59.37%), were able to adhere to polystyrene microplates, and five (15.6%) were strong adherent, fourteen (43.75%) were moderate adherent and thirteen (40.65%) were no or weak adherent (Figure 1).

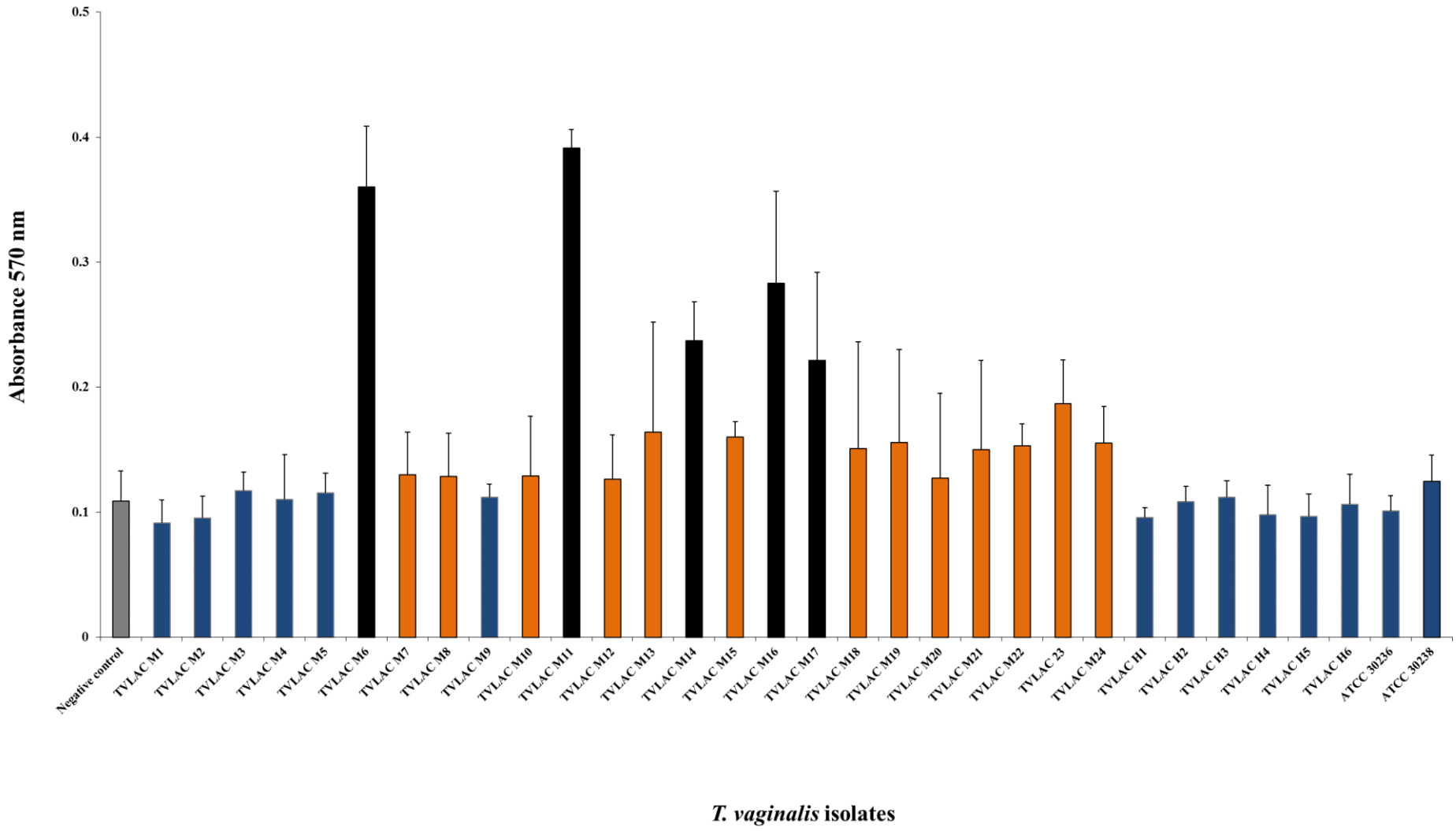

Absorbance 570 nm
0.5
0.4
0.3
0.2
0.1
0
Negative control
TVLAC M1
TVLAC M2
TVLAC M3
TVLAC M4
TVLAC M5
TVLAC M6
TVLAC M7
TVLAC M8
TVLAC M9
TVLAC M10
TVLAC M11
TVLAC M12
TVLAC M13
TVLAC M14
TVLAC M15
TVLAC M16
TVLAC M17
TVLAC M18
TVLAC M19
TVLAC M20
TVLAC M21
TVLAC M22
TVLAC 23
TVLAC M24
TVLAC H1
TVLAC H2
TVLAC H3
TVLAC H4
TVLAC H5
TVLAC H6
ATCC 30236
ATCC 30238
T. vaginalis isolates

Figure 1. Comparison of adherence levels to plastic among large number of *T. vaginalis* fresh clinical and ATCC isolates. The *T. vaginalis* isolates were classified as following: no or weak adherent isolates, represented in blue bars; moderate adherent isolates in orange bars and strong adherent isolates represented in black bars. All experiments were evaluated by visual inspection after washed microplates and before stained with crystal violet. It was observed in the strong adherent isolates a dense layer of trophozoites in amoeboid form covering the entire bottom of the well; in the isolates with moderate adhesion trophozoites in amoeboid form were observed in some spaces in the layers; in opposite, isolates classified as no or weak adherent presented only few trophozoites in amoeboid form. The results are the average and standard deviation from five independents experiments in triplicate. The bars are the value of crystal violet absorbance readings at 570 nm. In the negative control was used TYM medium without trophozoites represented in gray bar.

*3.*2 Trichomonas. vaginalis adherence to intrauterine devices and vaginal rings

Two strong plastic-adherent *T. vaginalis* isolates (TV-LACM6 and TV-LACM14) and one ATCC isolate 30236, that was unable to adhere to plastic, were used in this assay. The TV-LACM6 and TVLC-M14 isolates, with high capacity of adhere to plastic were also able to adhere in both IUD-threads and vaginal ring. On the other hand and expectedly, the ATCC isolate 3026 were less able to adhere to these devices (Figure 2). To further confirm the ability of TV-LACM6 and TV-LACM14 to adhere in IUD-threads and vaginal rings we used confocal microscopy assay. This capacity is clearly observed in figures 3A, 3C, and 4A, 4C, where both isolates were able to adhere in intrauterine devices and vaginal rings. Interestingly the adherence from trophozoites was highest in IUD-threads than vaginal ring.

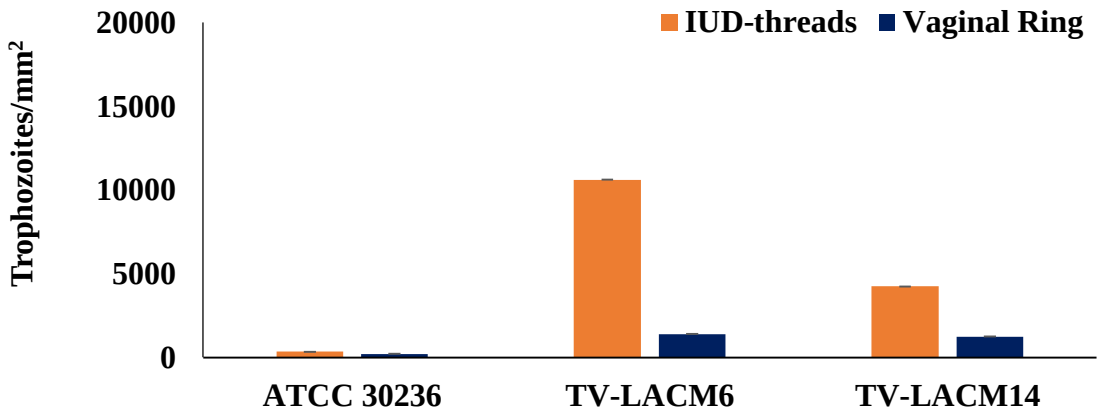


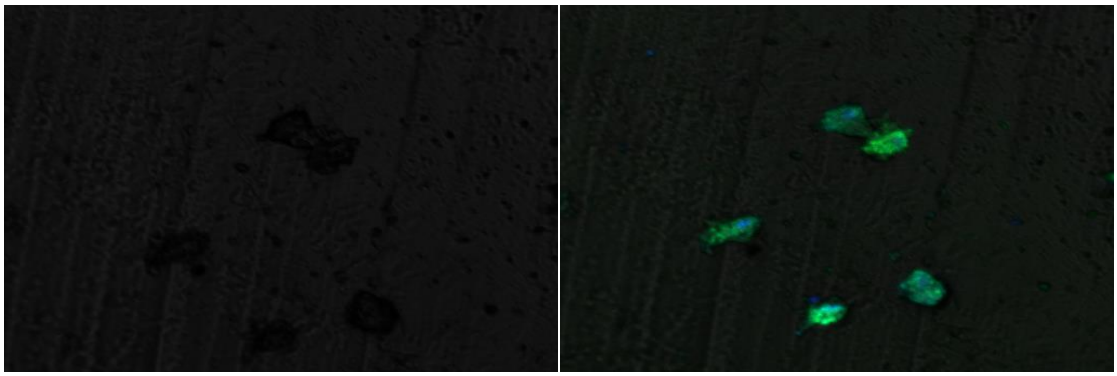
Figure 2. Adherence to intra-uterine device (IUD)-threats and vaginal ring from one **ATCC and two fresh *T. vaginalis* isolates.** The results are the number of trophozoites/mL of ATCC 30236, TV-LACM6 and TV-LACM14 by mm^2 from IUD-threats and vaginal ring. The bars represent the average and ± standard deviation from three independent experiments in triplicate.

3.3 Adherence to intrauterine devices and vaginal rings covered with metronidazole

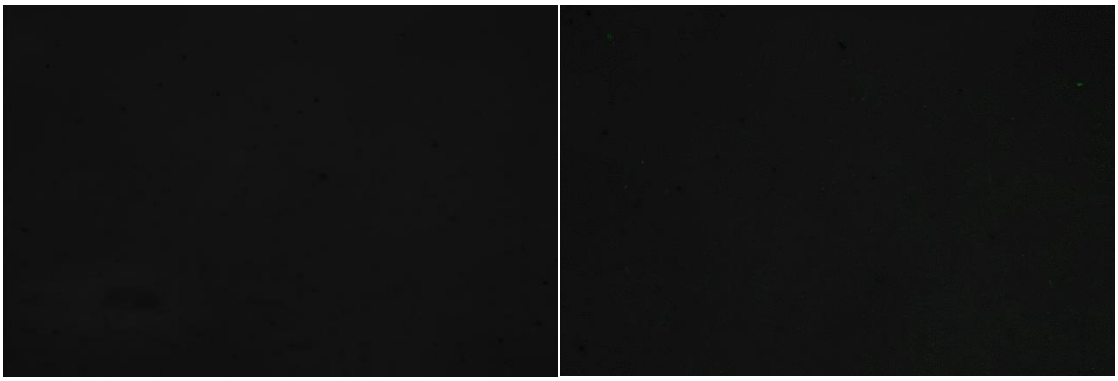
Since our results confirmed that the two strong adherent isolates to plastic were able to adhere in IUD-threads and in vaginal ring, we attempt to evaluate if there were any difference in adhesion level between metronidazole-device-covered and untreated device. Our findings of metronidazole-device-covered showed strong reduction in trophozoites adherence in both devices, IUD-threads and vaginal ring when compared with untreated as showed by confocal microscopy (Figures 3B, 3D and 4B, 4D).

Attached *T. vaginalis* in

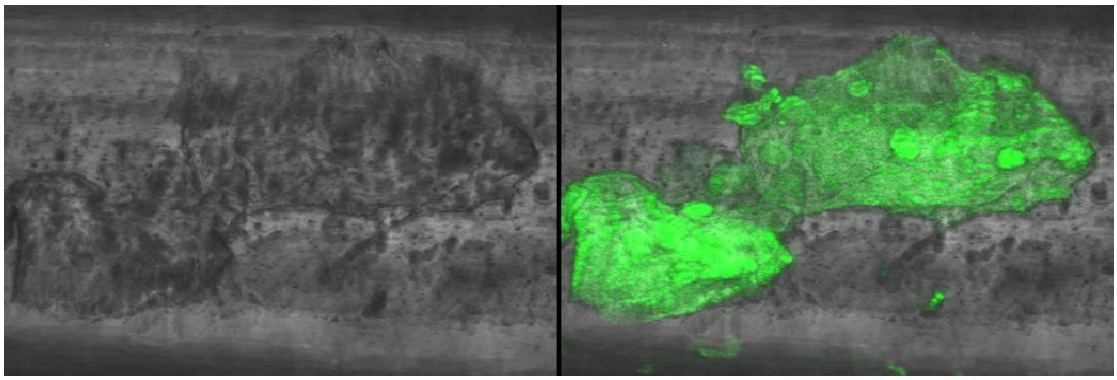
BRIGH **DAPI and**



Vaginal ring covered with 100mg/mL of



Attached *T. vaginalis* in



IUD-Threads covered with 100mg/mL of

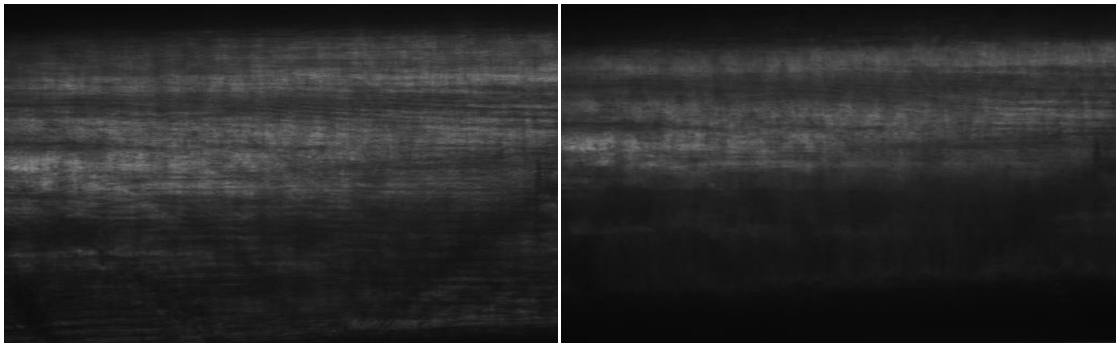
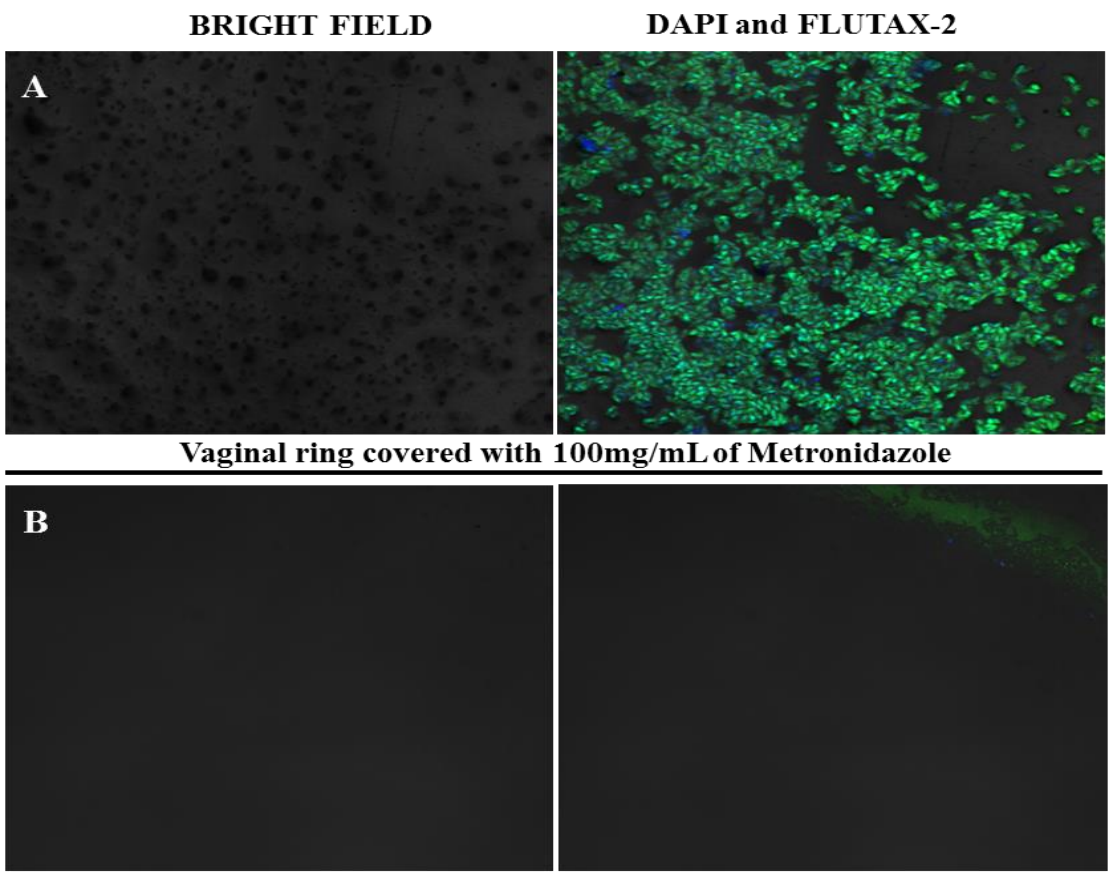


Figure 3. Confocal microscopy showing adherence by TV-LACM6 to vaginal ring and intra-uterine device (IUD)-threats. (A) Untreated T. vaginalis trophozoites attached to vaginal ring; (B) Vaginal ring covered with 100mg/mL of metronidazole; (C) Untreated T. vaginalis trophozoites attached in IUD-threats; (D) IUD-threats covered with 100mg/mL of metronidazole. Green and blue represent FLUTAX-2 and DAPI staining, respectively. Magnification X600.

T. vaginalis attached in Vaginal Ring



T. vaginalis attached in IUD-Threads

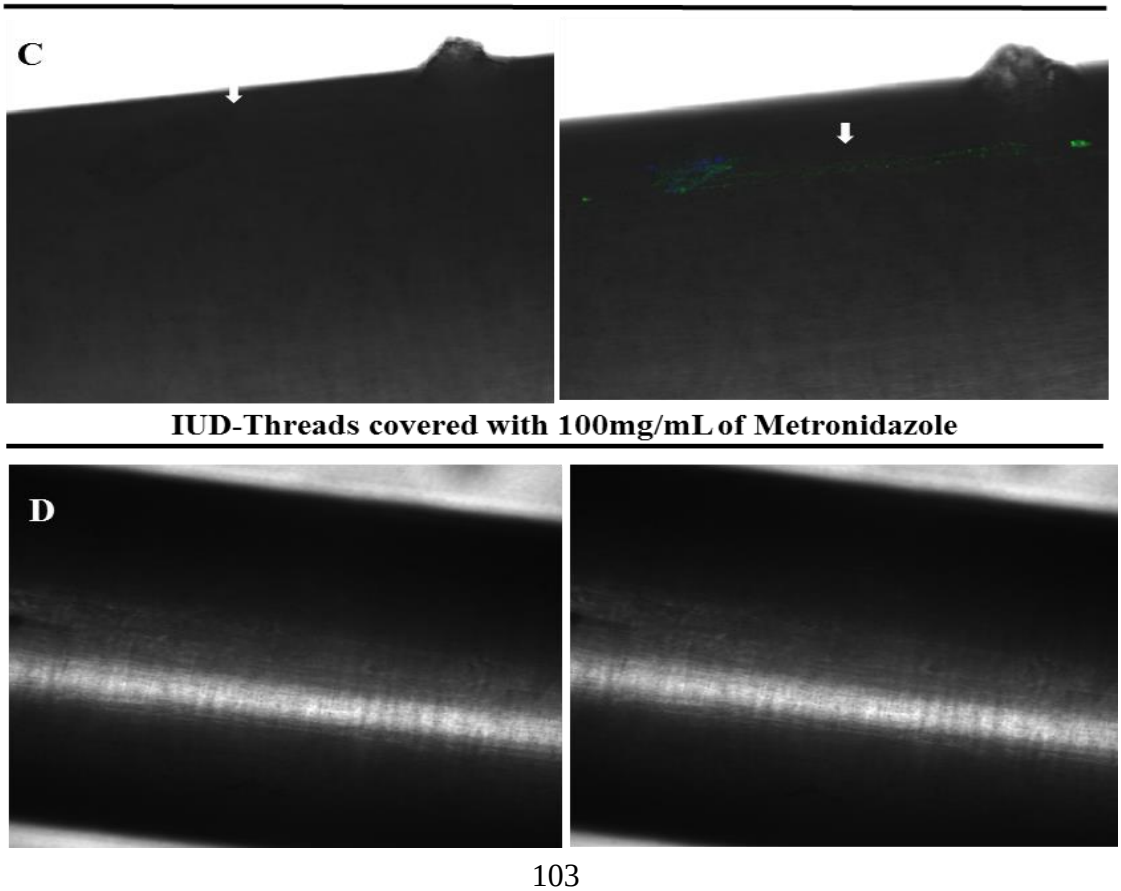


Figure 4. Confocal microscopy showing adherence by TV-LACM14 to vaginal ring and intra-uterine device (IUD) - threats. (A) Untreated T. vaginalis trophozoites attached in vaginal ring; (B) Vaginal ring covered with 100mg/mL of metronidazole; (C) Untreated T. vaginalis trophozoites attached in IUD-threats; (D) IUD-threats covered with 100mg/mL of metronidazole. Green and blue represent FLUTAX-2 and DAPI stainig, respectively. Magnification X600.

3.4 T. vaginalis adherence to polystyrene microplates after chemical treatment

To determine the role of different factors in adherence to plastic, trophozoites were chemically treated before the adherence assay. Two high adherent *T. vaginalis* isolates were used: TV-LACM6 and TV-LACM14. When the TV-LACM6 isolate was chemically treated it was possible to observe a reduction in adherence to plastic of 54.3%, 56.3%, 67.6%, 91.2% and 95.1% for colchicine, trypsin, cytochalasyn B, meta-periodate and MTZ treatments, respectively (Figure 5A). Similar results were found when TV-LACM14 isolate was chemically treated, the percent of reductions were 31.7%, 40.1%, 32.7%, 98.8% and 93.8% for colchicine, trypsin, cytochalasyn B, meta-periodate and MTZ, respectively (Figure 5A). After 30 min of chemical treatment, the parasites were counted and the cellular integrity was maintained. For both isolates TV-LACM6 and TV-LACM14, the number of trophozoites after chemical treatment was 1.0×10^6 trophozoites/mL (Figure 5B), the same initial density incubated. In all chemical treatments the trophozoites showed normal integrity and motility, except for MTZ treatment, it was observed changes in the morphology and decreased motility.

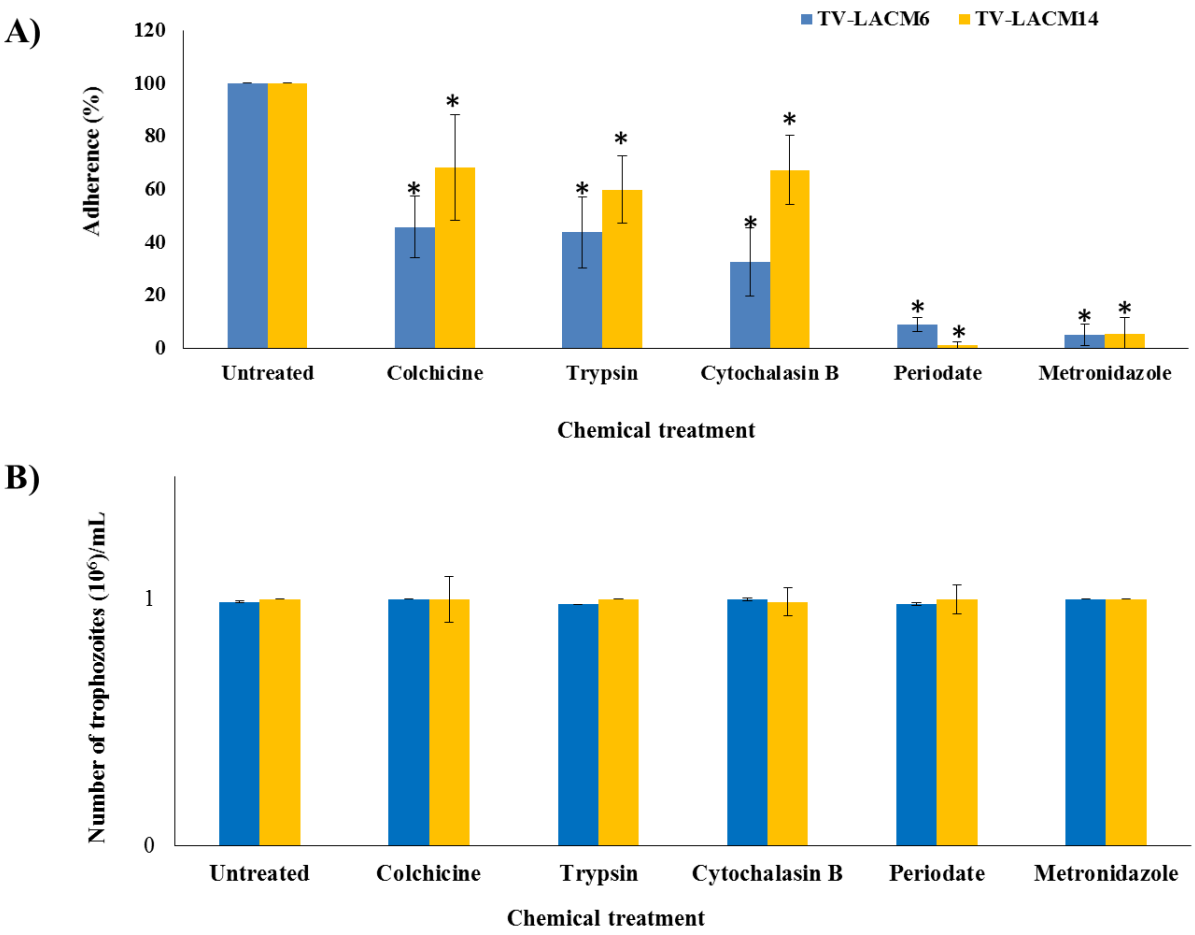


Figure 5. Comparison of adherence to plastic between untreated T. vaginalis and treated T. vaginalis with colchicine, trypsin, cytochalasin B, periodate, and metronidazole (MTZ). (A) The bars graph represents the average level of adherence by T. vaginalis TV-LACM6 and TV-LCM14 after chemical treatment; (B) The bars represent the number of trophozoites/mL of TV-LACM6 and TV-LCM14 counted at 30 min after chemical treatment. Adherence level of untreated T. vaginalis was normalized to 100% for control comparative propose. The bars represent the average and ± standard deviation from five independent experiments in triplicate. Statistical analyses using one-way ANOVA were performed; the level of significance was also determined by the Bonferroni method comparing all groups versus control. An asterisk above the bars (*) indicates the p values were less than 0.05.

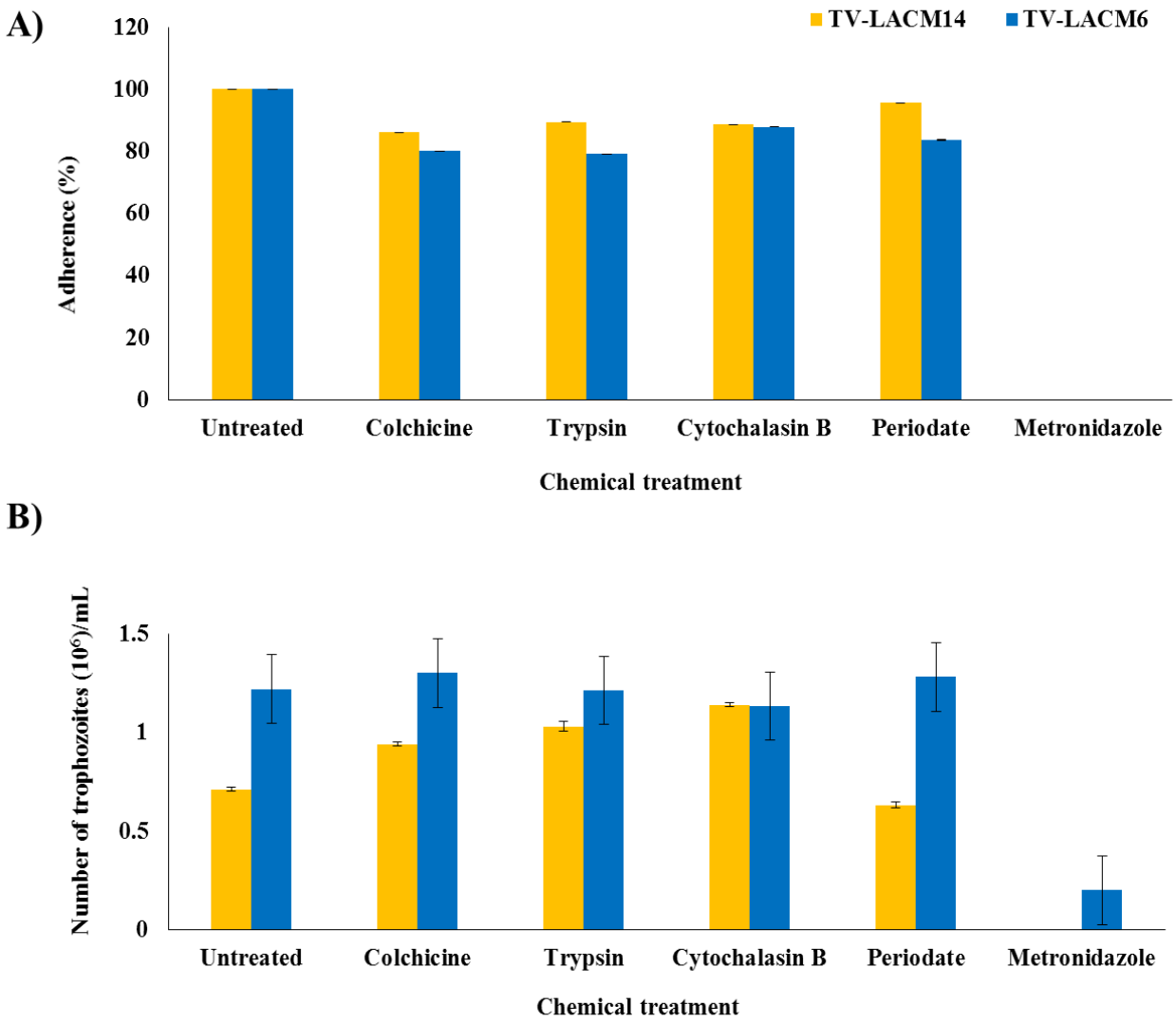


Figure 6. Comparison of adherence to plastic between untreated T. vaginalis and treated with colchicine, trypsin, cytochalasin B, periodate and metronidazole (MTZ), after removal of the chemical treatment. (A) The bars showing the average level of adherence by T. vaginalis TV-LACM6 and TV-LACM14 after 24 hours of incubation of the aliquot obtained after chemical treatment. (B) The bars represent the number of trophozoites/mL of TV-LACM6 and TV-LACM14 counted at 24h of an aliquot incubated after chemical treatment. The results were expressed by percentage of adherence when compared with untreated trophozoites, the bars showed the average and ± standard deviation from five independent experiments in triplicate for each condition. Adherence level of untreated T. vaginalis were normalized to 100% for comparative propose. As showed in Figure 6B, after removal of the drug treatment the number of trophozoites reached log growth phase and maintained the integrity and viability (Figure 6B) as well as it could be observed that the adherence ability was restored, except among isolates treated with metronidazole, as expectedly.

3.5 Trichomonas vaginalis cytodherence to HMVII after chemical treatment

To analyse the role of LPG, cytoskeletal parasite compounds, and surface molecules, the *T. vaginalis* isolates were pre-treated with chemical compounds before the cytoadherence assay. When the TV-LACM6 isolate was pre-treated with colchicine, trypsin, cytochalasin B and meta-periodate the cytoadherence reduction was 46.8, 58.1, 61.8, and 67%, respectively (Figure 7). In addition, there was little difference in the profile reduction when the TV-LACM14 was subjected to the same treatment; moreover, there are not statistical difference in these results. The cytoadherence reduction was 70, 44, 65, and 81% for colchicine, trypsin, cytochalasin B and meta-periodate, respectively (Figure 7).

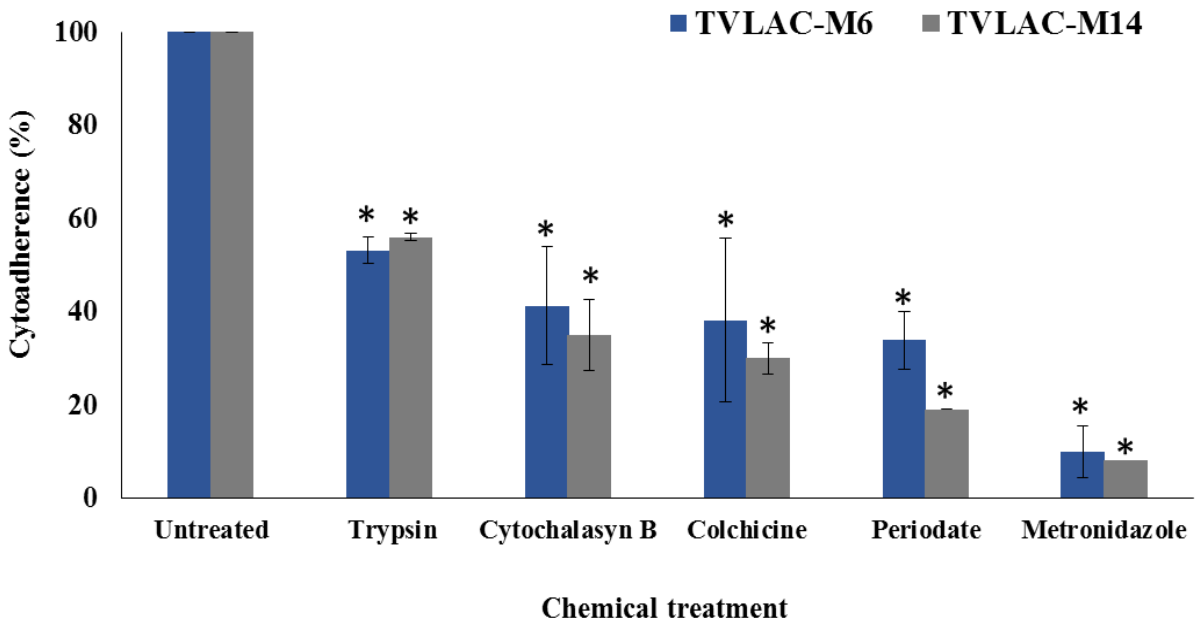


Figure 7. Comparison of cytoadherence between untreated T. vaginalis and treated with colchicine, trypsin, cytochalasin B, periodate and metronidazole (MTZ) by TV-LACM6 and TV-LACM14. The blue bars showing the mean level of cytoadherence by T. vaginalis TV-LACM6 after chemical treatment. The gray bars showing the mean level of cytoadherence by T. vaginalis TV-LACM14 after chemical treatment. Adherence levels of untreated T. vaginalis

were normalized to 100% for comparative propose. The results were expressed by percentage of cytoadherence when compared with untreated trophozoites, the bars showed the average and ± standard deviation from five independent experiments in triplicate for each condition. Statistical analyses using one-way ANOVA were performed; the level of significance was also determined by the Bonferroni method comparing all groups versus control. An asterisk above the bars (*) indicates the P values were less than 0.05.

3.6 Trichomonas. vaginalis cytodherence to HMVII versus adherence after chemical treatment

In summary, the percent reduction of *T. vaginalis* adherence to plastic and epithelial cells by the chemical treatments results showed that only in the chemical treatment using meta-periodate it was possible to observe a clear pattern of reduction in adhesion to plastic. For both isolates studied, TV-LACM6 and TV-LACM14, the LPG oxidation significantly inhibited the adherence to plastic, condition that lead to a reduction in the number of attached parasites to less than 3.0 %. The inhibition of adhesion to VECs was lower when compared to the inhibition of adherence to plastic when the trophozoites were pre-treated with meta-periodate, especially for TV-LACM6 isolate. Considering all chemical treatments used it was observed a wide variability (31.7 to 98.8 %) in the percentage of reduction for both cell attachment and adhesion to plastic.

3.7 Relative gene expression of four adhesins genes using qRT-PCR analysis

We utilized qRT-PCR to evaluate the relative expression of four adhesins genes (Ap33, Ap51, Ap65 and Ap120) in two isolates classified by us as strong adherent to plastic. All four adhesins were more expressed when the cytoadherence process was evaluated (Figure 8) in both isolates TV-LACM6 (Figure 8A) and TV-LACM14 (Figure 8B) ($P < 0.005$). The opposite phenomenon was observed in adhesins genes expression in adhered trophozoites in plastic, for all adhesins genes the relative expression level

was lowest than found in cytoadherence process. These findings suggest that adhesins proteins were involved only in cytoadherence process and not in the adhesion to plastic.

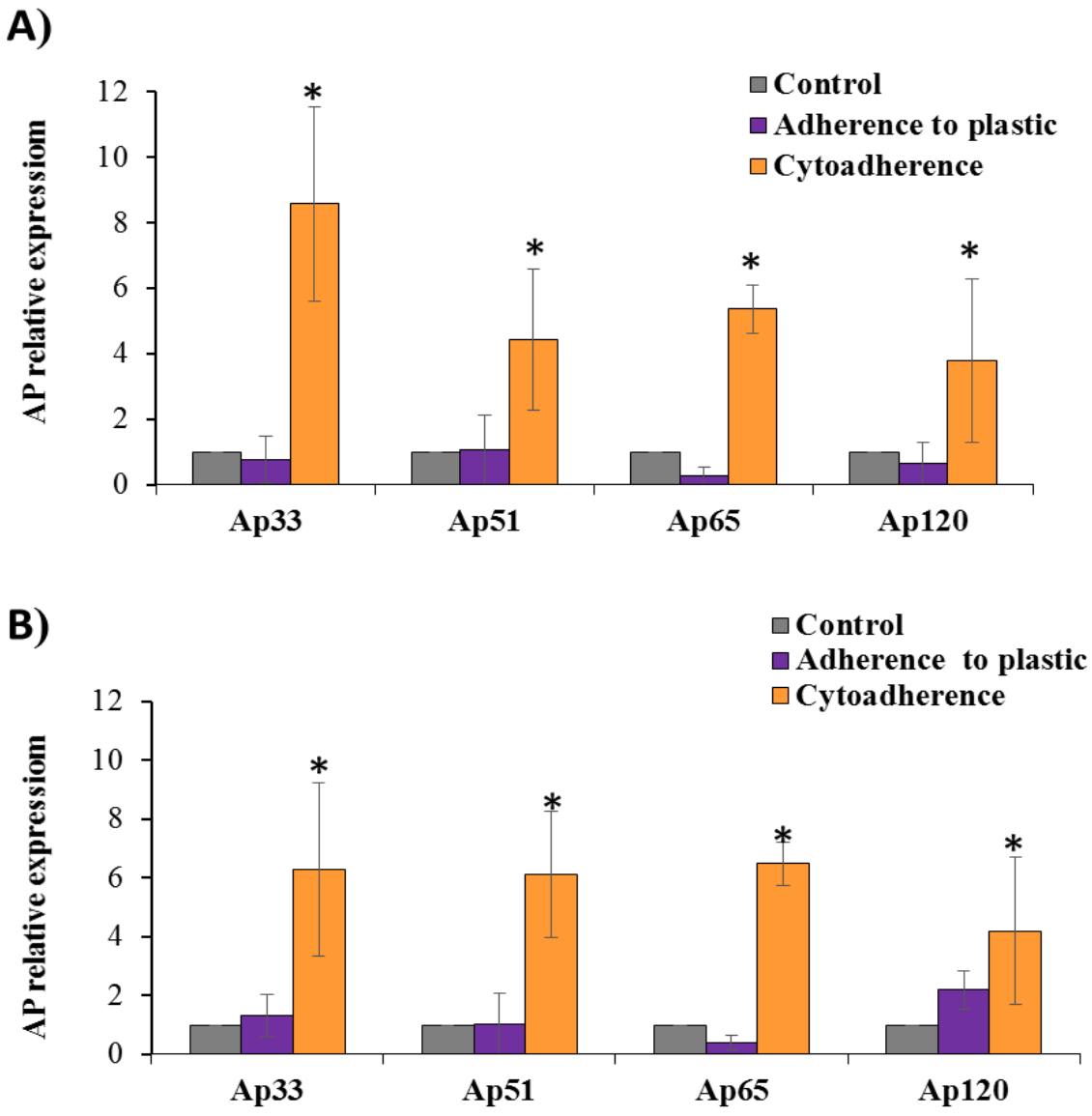


Figure 8. The relative gene expression of Ap33, Ap51, Ap65 and Ap120 in attached T. vaginalis trophozoites in plastic and VECs. (A) The relative expression of four adhesion genes by TV-LACM6 in trophozoites adhered in plastic represented by blue bars and trophozoites adhered in VECs represented by orange bars; (B) The relative expression of four adhesion genes by TV-LACM14 in trophozoites adhered in plastic represented by blue bars and trophozoites adhered in VECs represented by orange bars. The gray bars represent the not adhered control.

The bars represent the average ± SD of three biological replicate samples that were analyzed in three technical replicates to each experimental condition. The relative change in gene expression was analyzed using the 2-ΔΔCt method using Actin gene as internal control. Statistical significance in the expression changes of adhesins proteins between attached and unattached T. vaginalis in both processes of adherence to plastic and cythoaderence was calculated using one-way ANOVA; the level of significance was also determined by the Bonferroni method comparing all groups versus control ($P < 0.05$).

4. Discussion

Our results indicate that the capacity of *T. vaginalis* isolates to adhere in plastic is widespread, since most of isolates, more than 50 percent, were able to adhere to polystyrene microplates. This is an interesting result, considering that little is known about behaviour of adherence to plastic from large collections of *T. vaginalis* isolates. Previous studies have evaluated only one (GOLD and OFEK 1992) or two isolates (SILVA-FILHO, ELIAS, SOUZA, 1987; BASTIDA-COURCUERA, 2005) regarding this ability. Furthermore, another important result showed herein was the capacity of *T. vaginalis* to adhere to IUD-thread and vaginal ring. As expectedly, the strongest adherent isolates to plastic, TV-LACM6 and TV-LACM14, were also adherent to the medical devices tested, and in the other hand, the ATCC 30236 isolate, a non-plastic adherent, was unable to adhere to IUD-thread and to vaginal ring. Interestingly, these results agree with previously published data that showed one positive association between women using IUD and trichomonosis cases. Although somewhat speculative, *T. vaginalis* infection could be facilitated by the prolonged use of these devices, since the trophozoites may adhere to them in the vaginal cavity. (KAZEROONI and MOSALAEE, 2002). Therefore, we believe that the usage of these devices facilitates the establishment of *T. vaginalis* infection; however, other aspects are also important to be considered such as the modification of vaginal microbiota when IUD or vaginal ring are used, as well as women that use these devices as contraceptive methods probably use less condom.

We next hypothesized that metronidazole covered device had profound effects on the adherence to gynecological devices. Our findings showed that when the IUD-threads and vaginal ring were covered with metronidazole a significant adhesion inhibition occurred, since in confocal microscopy the trophozoites absent was observed. It is important to highlight that these two contraceptive approaches (IUD and vaginal ring) are popular in contraception planning. The IUD is the second most popular contraceptive method used in world and this medical dispositive use may increase the risk of STD acquisition. (TRUSSELL, 2012; STEINER, TRUSSELL and JOHNSON 2007). Likewise, the use of vaginal rings has been preferred due to efficacy and adherence to therapy while potentially decreasing toxic side effects when compared to daily pill oral administration (Valenta, 2005). Our investigation opened the question on whether covered devices with metronidazole or others molecules could be used as auxiliary tools for *T. vaginalis* control.

Adherence of *T. vaginalis* to host cells is a major factor in host-parasite relationships; in addition, surface proteins are likely to play important roles in the initial adherence to mucosal tissue as well as the long-term survival of the parasite on mucosal surfaces. Thus, our research group attempt to understand the role of different molecules in adhesion to plastic and to cytoadherence in fresh clinical isolates using the chemical treatment and the analysis of adhesins gene expression.

Our initial approach was to evaluate the trophozoites behavior after chemical treatment, and we observed that meta-periodate treatment dramatically reduced the number of adherent parasites in the plastic surface. The effect of periodate in *T. vaginalis* adhesion suggests that the involvement of the major cell surface glyconjugate, the lipophosphoglycan (LPG), in plastic adhesion is essential. Bastida-Courcuera et al (2005) have demonstrated the important role of LPG in adhesion to plastic. However, treatment with drugs that affect cytoskeletal function in trichomonads (colchicine and cytochalasin B) were also able to reduce significantly the adherence to plastic, indicating, not unexpectedly, that the perturbation of cytoskeletal structures also efficiently impaired plastic adherence. As expected, the metronidazole-treated *T. vaginalis* trophozoites reduced dramatically the adherence to plastic, showing that parasites metabolic integrity is essential for the attachment.

The absence of adherence or cytotoxic effect of parasites treated with periodate in VECs was found by Gilbert et al (2000). These authors suggested that the adherence to human host cell is modulated by LPG. The same study affirms that the periodate treatment abolished the binding of *T. vaginalis* to human host cells, VECs and HeLa. On the other hand, Alderete and Garza (1985), stated that the periodate treatment had no effect on host cell parasitism using HeLa cells. Our findings, using HMVII cell line showed that periodate treatment significantly reduced the adhesion to epithelial cells, however, did not completely abolish the binding between the parasite and host cell. In addition, we observed that the reduction in cytoadherence seems to be associated with the isolate, since TV-LACM6 had a lower cytoadherence reduction rate than TV-LACM14, when they were pre-treated with periodate.

In the same manner as occurred in adherence to plastic, the chemical treatments with colchicine, cytochalasin B and trypsin were also able to reduce significantly the cytoadherence, consequently, this perturbation of cytoskeletal structure and changes in the surface molecules lead to inefficient host-parasite interaction. We also observed that there were differences among the isolates in response to chemical treatments; therefore, it was not possible to determine the main mechanism involved in cytoadherence using only chemical treatment. However, it was observed that in the cytoadherence LPG also plays an important role.

Considering the involvement of adhesins for both adhesion to plastic and cytoadherence processes, our data suggest that the four-adhesins protein studied herein play important roles only in the cytoadherence. In opposite, in the plastic adherence the adhesin proteins appear to be not essential. These findings are very interesting, since these results are in agreement with previous study that showed that LPG play a important role in adherence to plastic (Bastida-Courcuera et al,2005). In the same way, our finding suggest that cytoadherence of the parasite is thought to be mediated by trichomonad surface proteins, studied here, AP33, AP51, AP65, and AP120, and the role of these proteins in cell adherence has been characterized, although the receptors in the host cell have not yet been identified (ALDERETE and GARZA, 1985; Engbring and Alderete, 1998; Alderete et al 1995; Moreno-Brito et al 2005). In a different way, the LPG was showed as main molecule in the adherence and cytotoxicity of parasites to human cells

(BASTIDA-COURCUERA et al, 2005), and the receptors for LPG, galectin 1 and 3 on vaginal and cervical epithelial cells was characterized. (OKUMURA et al, 2008; FICHOROVA et al, 2016).

Overall, our results reaffirm the importance of *T. vaginalis* as an important pathogen among women in reproductive age, since the trophozoites were able to adhere in gynecological devices and this fact could facilitate the parasite infection in the vaginal cavity. Furthermore, our chemical treatment data also suggest that for both adhesion to plastic and cytoadherence processes more than one factor is involved, and this has amplified the difficulty in the search for human host cell molecules involved in parasite attachment. Finally, our results indicate some differences between cytoadherence process and adhesion to plastic, subsequently the high level of adhesins expression was observed only in trophozoites attached to VECs. However, the LPG appear be important in both processes, since the oxidation of LPG similarly and significantly reduced the adherence and cytoadherence.

Acknowledgements

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PARTE III

III.1 DISCUSSÃO GERAL

A infecção por *T. vaginalis* trata-se da DST de origem não viral mais frequente no mundo, segundo os últimos dados da OMS, 276 milhões de novos casos são registrados anualmente (WHO, 2012). Graves complicações podem estar associadas à tricomonose, incluindo doença inflamatória pélvica, infertilidade, predisposição ao câncer cervical e de próstata, baixo peso em recém-nascidos e, de forma importante, *T. vaginalis* tem sido associado à aquisição e transmissão do HIV. (MILLER e NYIRJESY, 2011; PETRIN et al, 1998; SCHWEBKE e BURGESS, 2004; POOLE e MCCLELLAND, 2013). A base do tratamento da tricomonose é o uso dos 5-nitroimidazóis (únicos fármacos aprovados pelo FDA, *Food and Drug Administration, USA*) sendo o metronidazol o fármaco de escolha. A existência de isolados resistentes ao metronidazol tem sido mostrada, os casos de resistência chegam até 9,6% das infecções. (SCHWEBKE e BARRIENTES, 2006). Nestes casos, a ausência de alternativas terapêuticas faz com que altas doses de metronidazol e tinidazol sejam administradas, consequentemente proporcionando o surgimento de efeitos adversos e persistindo na falha em erradicar o parasito. Considerando essa problemática, torna-se evidente a necessidade da busca por novos compostos com ação tricomonicida estruturalmente distintos dos 5-nitroimidazois. Sendo assim, o completo entendimento do *T. vaginalis*, incluindo as suas propriedades bioquímicas, os mecanismos moleculares e os fatores de virulência, que são fundamentais para o entendimento da biologia e da patogênese desse protozoário e sua relação com as células hospedeiras é fundamental para a investigação de novos alvos terapêuticos e para conduzir de forma racional a elucidação de novos agentes anti-*T. vaginalis.*

Neste contexto, o estudo da tricomonose de forma mais ampla vem despertando interesse, uma vez que contribui para compreensão da doença, assim como para orientação das políticas públicas de saúde. No sentido de colaborar para a caracterização dos números da epidemiologia desta DST, realizou-se um revisão sistemática da literatura buscando estudos de prevalência e incidência da tricomonose publicados entre 2009 a 2015. Os resultados encontrados neste estudo nos permitiram fazer as considerações seguintes.

Existem escassos estudos, com qualidade metodológica, quanto à prevalência e incidência da tricomonose. Assim, pouco se sabe sobre os números dessa doença, que

é, muitas vezes, silenciosa e causa complicações graves à saúde humana. Os melhores dados (quali e quantitativos) obtidos na revisão sistemática foram com relação aos Estados Unidos da América (EUA), país em que, nos últimos anos, foram publicados relevantes estudos, citando a tricomonose como uma doença que tem recebido pouca atenção pelas autoridades locais de saúde e cujas estimativas da doença naquele país são subestimadas. No Brasil, três estudos de prevalência com qualidade foram encontrados. Sob outro aspecto, nenhum estudo de incidência foi publicado. As estimativas da prevalência da tricomonose no Brasil variam de 2,6% em Uberlândia, MG, a 12,7% em Coari, no Amazonas. (GRAMA et al, 2013; ROCHA et al, 2014). Reafirma-se, portanto, a posição da tricomonose como uma doença parasitária negligenciada.

Desde a publicação do genoma de *T. vaginalis* (CARLTON et al, 2007), inúmeras abordagens têm sido possíveis para o estudo do parasito, tais como diversidade genética, mecanismos de patogenicidade e interações entre o parasito e o hospedeiro. Consequentemente, a base de dados com o genoma completo do *T. vaginalis* disponível facilitou a realização de estudos expressão gênica. (BRADIC et al, 2014).

A reação de transcrição reversa, seguida da reação da polimerase em cadeia de forma quantitativa - qRT-PCR é um método amplamente utilizado, constituindo-se em uma robusta ferramenta, devido a sua rapidez, sensibilidade, especificidade e, sobretudo pela medida precisa dos níveis de Mrna. (VANGUILDER, VRANA, FREEMAN, 2008; BRADIC et al, 2014; NAPOLITANO et al, 2014; NAPOLITANO et al, 2014). Inúmeras são as variáveis técnicas, no entanto, inerentes a esta metodologia, as quais podem levar a uma interpretação errônea dos dados. Entre as principais limitações descritas encontram-se a qualidade e a quantidade de RNA acrescentado na reação de PCR, erros de transcrição reversa e erros de pipetagem. (KOZERA e RAPACZ, 2013). Para minimizar ou evitar as influências dessas variáveis emprega-se a normalização do gene estudado com um *housekeeping gene* ou um gene normalizador. Esta abordagem permite regular variações não biológicas, desde que ambos os genes (teste e normalizador) sejam expostos às mesmas variações técnicas. (REDDY et al2013; KOZERA e RAPACZ, 2013). Muitos genes denominados como *housekeeping* apresentam, todavia, instabilidade em seu nível de expressão constitutiva. (KOZERA e RAPACZ, 2013; HUGGETT et al, 2005; MAROUFI et al, 2010). Neste contexto, antes

da utilização de um gene como referência é fundamental que a sua expressão constitutiva seja avaliada, ou seja, a sua estabilidade. (VANDESOMPELE et al, 2002). Nosso grupo de pesquisa tem buscado entender os mecanismos de patogenicidade da tricomonose e, a cada etapa da nossa jornada, buscamos ampliar o uso de novas ferramentas especialmente para avaliar o papel de proteínas na relação parasito-hospedeiro. O objetivo principal deste estudo foi avaliar o processo de adesão às superfícies abióticas e a citoaderência. Para tanto, tornou-se necessário validar os genes de referência para estudos de expressão gênica para o *T. vaginalis.*

A validação de genes deu origem a um artigo que foi o primeiro relato na literatura científica de validação de genes normalizadores para *T. vaginalis.* Nesse estudo foram selecionados nove genes candidatos a normalizadores. Os trofozoítos foram cultivados em condições de restrição e de suplementação de nutrientes, sendo os níveis de mRNA quantificados usando qRT-PCR.

A estabilidade e o número de genes de referência necessários foram determinados utilizando-se os algoritmos *geNorm* e *NormFinder.* Os resultados demonstraram que apenas dois genes de referência já são suficientes para normalizar a expressão gênica, nessas condições de cultivo. Os genes mais estáveis foram *α-tubulina, actina* e *DNATopII.* Por outro lado, os genes mais instáveis foram *GAPDH* e *β-tubulina,* os quais são os mais amplamente utilizados como normalizadores em *T. vaginalis.*

Para validação dos genes de referência selecionados o nível de expressão da *piruvato:ferredoxina oxidoredutase* (PFOR) foi quantificado em trofozoítos tratados com sulfato ferroso usando *DNATopII, α-tubulina, actina GAPDH* como genes de referência. Nossos resultados confirmaram que a expressão relativa da *PFOR* foi aumentada quando utilizados *α-tubulina, actina* e *DNATopII* como genes de referência. Por outro lado, níveis reduzidos na expressão da *PFOR* foram observados quando *GAPDH* foi utilizado como normalizador. Estes resultados confirmam a necessidade da utilização de um gene de referência mais estável para obtenção de dados confiáveis. Para revalidar nossos resultados ampliamos nossos ensaios, utilizamos dois isolados frescos, TV-LACM6, selecionado por ser forte aderente ao plástico e apresentar elevada citotoxicicidade para células epiteliais vaginais e por ser infectado com *T. vaginalis* vírus (TVV) espécie (TVV 1) e com *Mycoplasma hominis* (Becker et al, 2015). Usamos

também o isolado TVLAC-H4 proveniente de um hospedeiro do sexo masculino e infectado com quatro diferentes espécies de TVVs (TVV 1, 2, 3, and 4) (Becker et al, 2015). Analisamos também a expressão da *PFOR* em trofozoítos tratados com diferentes concentrações de sulfato ferroso, bem como em diferentes tempos de incubação.

Finalmente, confirmamos a eficiência dos genes de referência mais estáveis ao obtermos resultados consistentes na expressão da *PFOR* de acordo com a concentração de sulfato ferroso utilizada, bem como com o tempo de exposição ao tratamento. Portanto, nossos resultados demonstraram que *DNATopII*, *α-tubulina* e *actina*, genes mais estáveis, levaram à correta interpretação dos dados, no entanto, quando *GAPDH* foi utilizado a expressão da *PFOR* foi subestimada levando ao erro de interpretação do dado.

Como principais considerações desse estudo apontamos para validação de nove genes de referência, os quais poderão servir como ponto de partida para diferentes estudos de expressão gênica em *T. vaginalis.* Outros aspectos importantes foram observados: *α-tubulina, actina* e *DNATopII* foram os genes que apresentaram maior estabilidade em sua expressão, na maioria das amostras testadas, isto é diferentes condições de cultivo. Por outro lado, *GAPDH* e *β-tubulina,* os mais usados genes de referência em estudos envolvendo *T. vaginalis* foram os mais instáveis. Além disso, nossos resultados sugerem que o uso de apenas dois genes *α-tubulina* e *actin* são suficientes para obter resultados confiáveis de expressão gênica. Nesse sentido, os dados obtidos com a validação dos citados genes de referência são importantes, tanto para o nosso grupo de pesquisa, quanto para a comunidade científica, uma vez que se trata de um importante ponto de partida para outros estudos de expressão gênica e de validação de novos conjuntos de genes de referência, em diferentes condições de cultivo.

O *T. vaginalis* é um patógeno extracelular e a citoaderência, ou seja, a aderência às CEVs do hospedeiro é fundamental para o processo de colonização e instalação da infecção. (ALDERETE et al, 1995; BASTIDA-CORCUERA et al, 2005). Trata-se de um processo complexo que envolve as proteínas de superfície, glicoconjugados e proteínas do citoesqueleto (FIGUEROA-ÂNGULO et al, 2012).

As adesinas de superfícies são proteínas exaustivamente estudadas como as principais moléculas envolvidas na adesão dos trofozoítos às células hospedeiras.

(ENGBRING e ALDERETE, 1998; KUCKNOOR et al, 2005; MORENO-BRITO et al, 2005). De acordo com Alderete e Garza, (1985), Engbring e Alderete (1998), Kucknoor et al, (2005) e Moreno-Brito et al (2005) existem cinco adesinas denominadas AP23, AP33, AP51, AP65 e AP120, as quais são expressas por famílias multigenes e foram identificadas e caracterizadas como proteínas multifuncionais, com funções dependentes da localização. (FIGUEROA-ÂNGULO et al, 2012; ALDERETE et al, 1995; ENGBRING e ALDERETE, 1998; MORENO-BRITO et al, 2005).

O *T. vaginalis* é coberto por um denso glicocálice, o qual tem como principal componente o lipofosfoglicano (LPG), que é o mais profuso polissacarídeo de superfície (Bastida-Corcuera et al, 2005). O LPG tem alta concentração na superfície do parasito e constitui-se numa molécula complexa, possuindo uma âncora de fosfoceramida inositol e um núcleo glicano fosforilado, composto de 50 a 54 resíduos de monossacarídeos, com uma composição diferente de outros parasitos. (SINGH, 1994). Segundo Bastida-Corcuera et al (2005), esses carboidratos de superfície são pouco explorados quanto ao seu papel na adesão. Entretanto, ao expor os trofozoítos de *T. vaginalis* ao periodato, que é um forte oxidante de polissacarídeos de superfície. (GILBERT et al, 2000), às enzimas que digerem o glicocálice (MIRHAGHANI et al 1998) ou a outros açúcares que competem para a ligação (GOLD e OFEK, 1992), houve significativa redução na capacidade de adesão às células mamíferas. Okumura e colaboradores em 2008, demonstraram que a galactina 1(Gal-1), que pertence à família das lectinas, é expresso pelas células do epitélio cervical e liga-se ao LPG do *T. vaginalis,* através de uma ligação dependente de carboidrato, ou seja, de galactose. (OKUMURA, BAUM, JOHNSON, 2008). Recentemente, Fichorova et al (2016) demonstraram que o LPG liga-se à galectina 1 e galectina 3 na superfície das células epiteliais vaginais e cervicais para modular a imunidade. Os carboidratos de superfície celular do parasito são considerados o principal mecanismo envolvido na aderência a superfícies de vidro e ao plástico. (PETRIN et al, 1998; BASTIDA-CORCUERA et al, 2005). No entanto, pouco já foi estudado sobre adesão de *T. vaginalis* em superfícies abióticas.

Com base nesse panorama, avaliamos o processo de patogênese da tricomonose por meio da adesão ao plástico, em trinta e dois isolados frescos de *T. vaginalis.* Desses,

59 (35%) aderiram, comprovando que a capacidade de adesão a uma superfície abiótica é uma característica comum em *T. vaginalis*. Após, foram escolhidos dois isolados de *T. vaginalis*, dentre os fortes aderentes, para serem usados como modelo de estudo dos processos de adesão às superfícies abióticas e de citoaderência: TV-LACM6 e TV-LACM14. Os trofozoítos foram tratados quimicamente com moléculas capazes de oxidar o LPG, de provocar alterações no citoesqueleto e de alterar outras moléculas de superfície (meta-periodato, colchicina, citochalasina B, tripsina e metronidazol, respectivamente). Como consequência dos tratamentos químicos, observamos que os isolados TV-LACM6 e TV-LACM14 reduziram os seus níveis de adesão às CEVs e ao plástico, o que comprova que o *T. vaginalis* realiza a patogênese por meio de adesão e citoaderência, fazendo uso de múltiplos mecanismos, os quais envolvem a participação do LPG e de proteínas de membrana. O nível de expressão de quatro adesinas de superfície em trofozoítos aderidos ao plástico e citoaderidos demonstrou um aumento nos níveis de expressão nos quatro genes somente quando os trofozoítos estavam aderidos às células epiteliais, estes resultados sugerem que as adesinas de superfície apresentam especificidade para as células do hospedeiro, pois, não apresentaram nenhum aumento em sua expressão quando os trofozoítos estavam aderidos ao plástico. Por outro lado a oxidação do LPG demostrou expressiva redução na desão ao plástico sugerindo um importante envolvimento dessa moléclula na adesão às superfícies abióticas. Consequentemente, nossos resultados sugerem que o LPG pode ser o principal mecanismo envolvido na adesão ao plástico. Da mesma forma, na citoaderência o LPG também demonstrou um importante papel, pois, houve significativa redução na citoaderência em trofozoítos submetidos à oxidação do LPG, embora, com os percentuais menores quando comparado com a redução da adesão ao plástico.

Os isolados frescos de *T. vaginalis* TV-LACM6 e TV-LACM14 aderem ao DIU e ao anel vaginal. Por outro lado, o isolado ATCC 30236 apresentou adesão praticamente nula a tais dispositivos. Com esses resultados, sugerimos que o DIU e o anel vaginal poderão servir como facilitadores da tricomonose, pois, os parasitos podem aderir a esses dispositivos no ambiente vaginal. Outro aspecto importante observado foi que o recorimento do DIU e do anel vaginal com metronidazol impediu a adesão dos trofozoítos. Esse resultado chama atenção para a

possibilidade do recobrimento destes dispositivos como medida de controle da disseminação do *T.vaginalis,* bem como para prevenção da doença.

III.2 CONCLUSÕES GERAIS

Os resultados obtidos no desenvolvimento desta tese permitem as seguintes conclusões:

Do artigo I

a) Os resultados obtidos na revisão sistemática demontraram que existem escassos estudos, com qualidade metodológica, quanto à prevalência e incidência da tricomonose;

b) Pouco se sabe sobre os números dessa doença, que é, muitas vezes, silenciosa e causa complicações graves à saúde humana;

c) Os melhores dados (quali e quantitativos) obtidos na revisão sistemática foram com relação aos Estados Unidos da América (EUA);

d) Esses resultados reafirmam o papel da tricomonose como uma doença que tem recebido pouca atenção pelas autoridades de saúde.

Do artigo II

a) Os genes de referência *actina, α-tubulina* e *DNATopII* foram os genes mais estáveis em condições de restrições de nutrientes;
b) Apenas dois genes de referência já são suficientes para normalizar, nessas condições de cultivo, a expressão gênica.
c) Nossos resultados confirmaram que não existe um gene de referência universal e antes de estudos da expressão gênica existe a necessidade da otimização de genes para serem utilizados como referência;
d) Finalmente, estes resultados são os primeiros descritos na literatura científica e servirão de base para novos estudos de expressão de genes em *T. vaginalis.*

Do artigo III

a) O *T. vaginalis* tem uma ampla capacidade de aderir às superfícies abióticas, uma vez que um número expressivo de isolados frescos aderiram às microplacas;

b) O *T. vaginalis* aderiu ao fio do DIU e ao anel vaginal, reforçando o papel dessa DST nas mulheres em idade reprodutiva;
c) O recobrimento dos dispositivos ginecológicos com o metronidazol é capaz de impedir a adesão dos trofozoítos;
d) Supomos que os dispositivos ginecológicos poderão atuar como facilitadores da infecção para os *T. vaginalis;*
e) Confirmamos que a adesão às superfícies abióticas e a citoaderência envolve múltiplos mecanismos;
f) O LPG tem um papel importante tanto na aderência quanto na citoaderência;
g) As adesinas de superfície parecem estar relacionadas somente com a citoaderência.

PARTE IV

IV.1 PERSPECTIVAS

Alguns aspetos sobre a adesão e a citoaderência merecem ser investigados futuramente:

a) Avaliar a expressão das adesinas de superfície em número maior de isolados;
b) Avaliar a adesão ao DIU e ao anel vaginal em outros isolados de *T. vaginalis;*
c) Identificar compostos com ação anti-*T. vaginalis* para recobrir as superficies do DIU e do anel vaginal considerando a crescente resistência de isolados clínicos de *T. vaginalis* ao metronidazol e tinidazol.

PARTE V

V.1 REFERÊNCIAS

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PARTE VI

VI.1 ANEXO

Anexo I: Artigos Científicos Publicados no Período de Vigência do Doutorado
(março 2012-março 2016)

1. Frasson AP, Santos O, Meirelles LC, Macedo AJ and Tasca T (2016) Five putative Nucleoside Triphosphate Diphosphohydrolases (NTPDases) genes are expressed in *Trichomonas vaginalis.* FEMS Microbiolo lett.

2. Becker DD, Santos O, Frasson AP, Rigo VG, Macedo AJ and Tasca T (2015) High rates of Double Stranded RNA Viruses and Mycoplasma hominis in *Trichomonas vaginalis* Clinical Isolates in South Brazil. Infection Genetics and Evolution.

3. Primon BM, Rigo VG, Frasson AP, Santos O, Smiderle L, Almeida S, Macedo AJ and Tasca T (2015) Modulatory effect of iron chelators on adenosine deaminase activity and gene expression in *Trichomonas vaginalis.* Memórias do Instituto Oswaldo Cruz.

4. Scopel M, Santos O, Frasson AP, Wolfrainer A, Tasca T, Henriques AT, Macedo AJ (2013) Anti *Trichomonas vaginalis* activity of marine associated fungi from the South Brazilian Coast. Experimental Parasitology.

5. Frasson AP, Santos O, Duarte M, Tretin SD, Brandt GR, Silva AG, Siva, MV, Tasca Tiana, Mecedo AJ (2010) First report of anti *Trichomonas vaginalis* activity of the medicinal plant Polygala decumbens from the Brazilian semiarid region, Caatinga. Parasitology Research.