Trichomonas vaginalis, A Model Mucosal Parasite

By

John F. Alderete, Ph.D.

Department of Microbiology

The University of Texas Health Science Center

San Antonio, TX 78284-7758 USA

Running Title: *T. vaginalis* virulence and pathogenesis

Keywords: Cysteine Proteinases, Cytoadherence, Environmental Regulation, Immune Evasion, Nutrient Acquisition, Pathogenesis, *Trichomonas vaginalis*

Address for correspondence:
John F. Alderete, Ph.D., Department of Microbiology, UTHSCSA, 7703 Floyd Curl Drive, San Antonio, TX 78284-7758 USA, 210-567-6828, alderete@uthscsa.edu

Abstract: Trichomonosis is the vaginitis caused by the sexually transmitted protist, *Trichomonas vaginalis*. Infection of women, in contrast to men, by this flagellated protozoan is non-self-limiting and requires detection for elimination by drug therapy. Diagnosis of infected women is complicated by the wide variations in symptomatology. The parasite survives in the constantly changing and adverse environment of the female urogenital tract. Several mechanisms (Table 1) are employed by *T. vaginalis* to achieve successful host parasitism. The acquisition of nutrients from mucosal secretions or from hemolysis and cytotoxicity overcomes the immediate nutritional immunity imposed by the new environment. Recognition and binding to vaginal epithelial cells by adhesins that resemble metabolic enzymes is a step preparatory to infection. The evasion of immune responses, which can be trichomonacidal, is accomplished through masking of organisms by host proteins, shedding of trichomonad proteins into the secretions, and degradation of all immunoglobulin subclasses and complement. Parasites undergo phenotypic variation between surface and cytoplasmic expression of a repertoire of immunogenic proteins to which antibody is directed. Moreover, the expression of virulence genes is under the control of parasite and host environmental factors.

CLINICAL ASPECTS OF TRICHOMONADS.

This brief section on clinical aspects of trichomonosis (this term now replaces trichomoniasis [1]) will highlight areas in which there is general appreciation regarding the complexity and multifactorial nature of the symptoms. Trichomonosis is now the number one, non-viral sexually transmitted disease worldwide [2]. Infection with *Trichomonas vaginalis* has major health consequences for women, including predisposition to HIV [3, 4], association with cervical cancer [5], and complications of pregnancy [6]. The vaginal epithelium is the primary site of infection. A hallmark and complication among patients with trichomonosis is the wide variations in symptomatology. While some patients are characterized as asymptomatic because of their individual perceptions, women with vaginitis experience a yellow-green discharge due to a leukocytic infiltration, abdominal pain, irritation, and discomfort [7-9]. The nature of the discharge is different from that seen for candidiasis and bacterial vaginosis [7]. The vagina and cervix of patients with trichomonosis may be erythematous and edematous, with general erosion of the cervical epithelium and punctate hemorrahages on the cervical wall termed “colpitis macularis” or “strawberry cervix” [7, 10]. Although present in only a few (2% to 5%) women, the strawberry cervix is nonetheless highly specific for trichomonosis [10]. Sharp abdominal pain documented among patients with trichomonosis may be indicative of infection in the upper urogenital region, possibly involving regional lymphadenopathy and salpingitis [11].

In contrast to women, men become infected following recent contact with an infected partner but, for unknown reasons, have a self-limiting infection [12]. Nongonococcal nonchlamydial urethritis, which disappears after treatment, is found among men infected with *T. vaginalis* [7, 13]. The presence of trichomonads in the prostate gland in men with trichomonosis has been reported. However, the role of infection in prostatis and/or in infertility or numerous other reported sequelae is unclear [7, 11].

OVERCOMING NUTRITIONAL IMMUNITY

The vagina is one of the most complex sites of infection for a mucosal pathogen. This host environment is constantly changing under the influence of the menstrual cycle. It is a nutrient-

limiting site that cannot promote the 4 to 6 hour generation time seen during in vitro growth of habituated parasites in a serum-based, trypticase and yeast extract complex medium [15]. It has been established that trichomonads survive a severe nutrient-limiting environment with a generation time of 150 hours [16], something approaching the in vivo situation. Importantly, how expression of virulence factors is affected by this extended generation time must be examined in comparison to the shorts times of batch culture. Infecting organisms are capable of being coated with host macromolecules [17], some of which contribute to the obligate nutritional requirements of the trichomonads and, therefore, pathogenicity. The lack of enzymes for synthesis of lipids or conversion and retroconversion of lipids [18] is overcome through receptor-mediated binding and uptake of lipoproteins found in serum or vaginal secretions [20] and of specific hemagglutination [19] followed by protease-mediate hemolysis [20, 21].

T. vaginalis requires high amounts of iron for optimal growth and multiplication [22] and for up-regulation of virulence genes [23, 24]. The association of iron within iron-binding and iron-containing proteins, some of which are under the control of the menstrual cycle [25], imposes a nutritional immunity upon these parasites, which is only partially overcome through binding of lactoferrin by the trichomonal receptor and removal of iron [24]. That hemolysis [20, 21] and cytotoxicity [26, 27] stimulate growth of *T. vaginalis* under iron-restrictive environments [24] indicates that intracellular iron sources such as cytochromes, ferritin and hemoglobin (heme) may also be iron sources. Such multiple iron-acquisition systems appear prerequisite for successful host parasitism and pathogenesis.

INTERACTIONS BETWEEN TRICHOMONADS WITH HOST CELLS AND TISSUES

T. vaginalis trophozoites recognize and bind (cytoadhere) epithelial cells preferentially using host cells in monolayer cultures as model systems [7, 9, 26-28]. Isolation of squamous vaginal epithelial cells (VECs) and intermediate epithelial and parabasal cells demonstrated the ability of trichomonads to readily cytoadhere to VECs [29]. The VECs are terminally differentiated cells under the control of hormones, and the normal desquamation of cells illustrates the dynamic nature of cytoadherence during the menstrual cycle. The complexity of VEC cytoadherence by trichomonads is evident by the signaling for dramatic morphologic transformation [30] that occurs within minutes after attachment (Fig. 1b); HeLa epithelial cells on monolayers, showing specificity in the signaling process, give no similar signal (Fig. 1a). Optimal cytoadherence requires the activity of cysteine proteinases that acts uniquely on the parasite surface in an unknown fashion [31]. Two cysteine proteinases that bind to epithelial cells and whose activity correlates with cytoadherence and cytotoxicity [32] appear to be involved in this complex sequence of events.

Receptor-ligand-type interactions are involved between trichomonads and epithelial cells [28-30]. The surface structures on VECs recognized by *T. vaginalis* organisms are unknown, although trichomonads possess surface laminin-binding proteins [33]. While laminin is not found on VECs, this finding may be relevant to parasite associations with basement membrane sites. Four trichomonad surface proteins have been identified as mediating cytoadherence [28]. The synthesis of the four adhesins was coordinately up-regulated by binding to epithelial cells [30] and by iron [23]. The increased amounts of adhesins were localized to the surface adjacent to the VEC surface [30]. Interestingly, only fresh clinical isolates, but not long-term-grown cultures, synthesized greater amounts of adhesins in response to iron [23]. It is likely that signaling for

increased synthesis of adhesins following contact with VECs utilizes pools of iron within trichomonads [24]. Contact-dependent cytotoxicity mediated by cysteine proteinases [26, 27, 32] of VECs and surface acquisition of iron-binding and iron-containing proteins as summarized above represent a part of the nutrient-acquisition pathways of *T. vaginalis*.

More recent work reveals that three of the four adhesins studied to date are each members of multi-gene families [34-36], and sequence analyses at both the nucleotide and amino acid levels revealed structural molecular mimicry of adhesins with known metabolic enzymes [37]. Analysis of the receptor-binding epitope for the adhesin AP33 identified the 24-amino acid binding domain with the ability to inhibit parasite associations with host cells [38]. It was noteworthy that purified enzymes with identity to the adhesins were incapable of inhibiting binding of the recombinant and natural adhesins to host cell surfaces nor of preventing trichomonal cytoadherence [34-38]. There is now a growing body of evidence for enzymes on the surface of microbial pathogens, as summarized previously [34-38], where the enzymes act as binding proteins for host ligands and adhesins for host cells.

Finally, it is reasonable to hypothesize that the non-self-limiting nature of trichomonosis cannot be solely explained by cytoadherence to VECs. Erosion of the vaginal epithelium as seen for colpitis macularis [7, 8, 10, 11] may allow access of parasites to the basement membrane and accompanying complex structures. Interestingly, the reports on the specific binding by *T.vaginalis* organisms to fibronectin [16] and laminin [33] may reflect associations with basement membrane sites. Future studies on the parasite ligands that bind these and other basement membrane components will clarify whether host parasitism involves sequential cytoadherence and association with basement membrane sites made available during infection.

EVADING TRICHOMONACIDAL IMMUNE RESPONSES

Cellular and humoral immune responses are evident in patients with trichomonosis [7-11]. The host is not protected against repeated infections with *T. vaginalis*, which are common, and treatment of previous trichomonosis appears a risk factor for a current infection. Although not found in all patients with trichomonosis, increased numbers of polymorphonuclear leukocytes can be readily detected in secretions. Interestingly, while both leukocytes and macrophages in addition to antibody and complement can eliminate parasites, it is clear that *T. vaginalis* has effectively neutralized the host immune surveillance system. Further, hydrogen peroxide-producing lactobacilli are considered protective normal vaginal flora [39].

As mentioned earlier, the parasite surface can be coated with host proteins found in vaginal secretions [16] leading to non-recognition by immune mechanisms. Shedding of large amounts of immunogens under conditions that do not result in lysis of trichomonads during growth and multiplication [40] represents a mechanism by which antibody and other immune effector molecules are neutralized. Analysis of vaginal secretions from patients revealed numerous trichomonad immunogens readily detectable by sera of patients and polyclonal rabbit anti-*T. vaginalis* serum, regardless whether immunogens are shed or are the result of lysis of parasites.

The numerous cysteine proteinases synthesized by *T. vaginalis* [41, 42] contribute significantly to immune evasion and pathogenesis. The cysteine proteinases are cytotoxic [26, 27, 31, 32] and hemolytic [20, 21]) and degrade basement membranes [43]. All subclasses of

immunoglobulins are susceptible to the trichomonad cysteine proteinases [43]. The numerous proteinases of *T. vaginalis* bathing the female urogenital environment is significant as evidenced by proteinases activity in vaginal secretions of patients but not in control, uninfected women or in women with sexually transmitted diseases other than trichomonosis [44]. Both vaginal secretions and sera of patients had detectable antibody to the numerous cysteine proteinases [45]. It is noteworthy that prior attempts by numerous laboratories to characterize accurately the vaginal antibody response may not have been successful because of the degradation of antibodies by the trichomonad proteinases. Future vaccines to protect mucosal surfaces must take into account the presence of the numerous parasite proteinases during trichomonosis.

Batch cultures and fresh clinical isolates of *T. vaginalis* organisms are readily killed by the alternative complement pathway [7, 46] yet survive during menstruation when complement in blood is available [46]. Parasites are resistant to lysis by the action of at least one cysteine proteinase induced by high iron growth conditions. The proteinase degrades C3 deposited on *T. vaginalis* surfaces [47]. This finding further shows the regulation of expression of proteinases by environmental factors, such as iron, and is consistent with the differential expression of activity among fresh clinical isolates examined immediately after purification from patients [42]. In vitro cultivation of *T. vaginalis* for assessing specific properties, such as resistance to complement or cytoadherence, may be problematic if the growth medium lacks environmental factors that regulate expression of virulence factors responsible for those properties.

The in vivo synthesis of the proteinases [42] must be controlled by environmental factors, such as iron [47], so as to modulate the number and amount of proteinases required at the particular site of infection at that time. Otherwise, uncontrolled synthesis of proteinases may produce unwanted cytopathology that would lead to host responses that could eradicate the parasite. Host reducing levels also regulate in vivo the activity of the trichomonad proteinases [48]. In contrast to the large amounts of a reducing agent (1 mM dithiothreitol) used to examine experimentally the activity of cysteine proteinases [41-44], the reducing environment of the vagina of patients with trichomonosis ranged from $\leq$10 μM to 40 μM [48], a range that produced differential and quantitatively distinct activation of proteinases. Lastly, hydrogen peroxide readily neutralizes the cysteine proteinases [48], showing the protective effect of lactobacilli normal flora [39]. However, displacement of the lactobacilli immediately following infection with *T. vaginalis*, possibly through internalization and degradation by proteinases, subverts this host protective effect.

The findings on the reducing level in vaginal secretions are of fundamental importance to understanding fully the contribution of the numerous cysteine proteinases of *T. vaginalis* to virulence and pathogenesis. Among the questions that require attention are: What is the level of reducing power during the course of the menstrual cycle and are certain times during the cycle more favorable to activation of proteinases? Is there a difference in reducing levels among ethnic groups, explaining the higher rates of trichomonosis among African American women, for example [49]? Do certain behaviors, such as smoking, that correlate with higher rates of trichomonosis [49] alter the vaginal reducing environment in favor of proteinase activation? Is symptomatology related to the amounts and activation of proteinases by the vaginal reducing environment? Is the self limiting nature of trichomonosis in males a function of the relative absence of a reducing environment?

PHENOTYPIC VARIATION AND dsRNA VIRUS DEFINE TWO TYPES OF *T. vaginalis* ISOLATES

Phenotypic variation for *T. vaginalis* was defined on the basis of surface versus cytoplasmic expression of a repertoire of high M_r immunogens [50, 51]. This discovery resulted from experiments aimed at understanding the reported extensive antigenic heterogeneity among *T. vaginalis* isolates [7, 9]. To understand the antigenic heterogeneity among trichomonal isolates, a library of monoclonal antibodies (mAbs) was generated. One mAb, called C20A3, recognized a highly immunogenic 270,000 dalton (270-kDa) protein (P270). Analyses by flow cytofluorometry and fluorescence-activated cell sorting of fresh clinical isolates gave patterns of fluorescence similar to that reported among isolates using polyclonal antiserum of animals immunized with parasites or with sera from patients with trichomonosis. It became evident from fluorescence experiments that two types of isolates occur naturally during infections with *T. vaginalis* [50-51; Table 2]. Type I isolates were comprised of homogeneous populations of non-fluorescent trichomonads that synthesize and express P270 only in the cytoplasm. In contrast, Type II isolates were heterogeneous with subpopulations of both fluorescent and non-fluorescent parasites. Sorting of fluorescent and non-fluorescent trichomonads of Type II isolates with C20A3 demonstrated that each purified subpopulation reverted to the opposite phenotype. This demonstrated the property of phenotypic variation for the Type II trichomonads [50, 51]. There is an equal distribution between Type I and Type II isolates based on examination of over 1,000 isolates from throughout the world. It was further demonstrated that both murine mAb and polyclonal antibody from patients reactive with P270 was lytic for trichomonads with surface P270 in a complement-independent fashion. Among infecting phenotypically varying isolates, $\leq$10 % of the trichomonads expressed P270 on the surface [51], suggesting the host environment either eliminates parasites with surface P270 or favors cytoplasmic expression.

The identification of the double-stranded (ds)-RNA virus within *T. vaginalis* organisms established a relationship between virus infection and phenotypic variation [52]. Loss of virus from parental Type II isolate organisms by batch culture [7, 9, 53] produced virus-minus progeny identical to Type I virus-minus isolate parasites. The virus is multi-segmented, possessing a tripartite dsRNA genome [53]. A family of small-sized satellite dsRNAs that reside within the virus-infected trichomonads shows the complexity of the virus-parasite relationship.

The *p270* gene for the representative phenotypically varying *T. vaginalis* isolate T068-II has been sequenced [54]. This *p270* gene has a 333-bp unit tandemly repeated at least 18 times, as suggested by earlier reports [7, 9], within which is the DREGRD epitope recognized by the C20A3 mAb [50, 54]. The non-repeat coding regions for the 5′- and 3′-ends were 69 nucleotides (23 amino acids) and 1183 nucleotides (395 amino acids), respectively. This single copy gene was up-regulated by low iron conditions, which paralleled surface expression of P270 for these virus-harboring, phenotypically varying isolates (Fig. 2). Growth of virus-minus isolate parasites under similar low iron conditions also increased amounts of *p270* transcripts but did not promote surface expression of P270, strongly suggesting that the virus contributes regulatory factors that allow for mobilization of P270 onto the trichomonad surface.

PARASITE AND HOST FACTORS INFLUENCE EXPRESSION OF TRICHOMONAD GENES AND ANTIGENIC HETEROGENEITY

Although the mechanism(s) by which the virus confers the ability to surface express P270 remains unknown, it is conceivable that the dsRNA virus encodes regulatory proteins that affect expression of trichomonad genes. In addition to undergoing phenotypic variation, Type II organisms synthesize by at least two orders of magnitude greater amounts of P270 than do virus-minus Type I isolates or virus-minus progeny derived from virus-infected parental isolates. Moreover, it has been established that the dsRNA virus influences growth kinetics and expression of proteins of virus-infected *T. vaginalis* isolates [55]. Furthermore, high resolution two-dimensional electrophoretic analyses of total proteins revealed that numerous (forty-seven) proteins were specifically expressed for a virus-positive isolate when compared to its virus-minus progeny counterpart. Almost an equal number (forty-one) of different proteins were expressed in the corresponding virus-minus progeny when compared to the virus-positive parental parasites. Equally noteworthy, qualitatively and quantitatively dissimilar cysteine proteinase patterns were seen between virus-positive isolates and virus-minus progeny organisms. Clearly, there is a strong association between virus infection and the presence and absence of parasite proteins that may be important in virulence and pathogenesis.

Iron is required for growth and multiplication of *T. vaginalis* [22]. The nutritional immunity imposed by the host would be impossible to overcome if trichomonads relied solely on the interaction of lactoferrin with its trichomonad receptor [24]. Given the established role of iron in regulating growth kinetics and expression of receptors for iron-binding and iron-containing proteins, adhesins, cysteine proteinases, and immunogens [15, 23, 24, 43], it is not surprising that trichomonads acquire iron from multiple sources. Iron and the dsRNA virus promote surface expression of P270. Low-iron-grown, virus-harboring parasites uniformly express P270 on the surface (Fig. 1), which is readily reversed by addition of iron, an environment that in turn favors synthesis and surface expression of adhesins. Thus, for Type II isolates, intracellular levels of iron mediates synthesis and/or alternating expression of at least to groups of proteins on the surface of trichomonads. In addition to specific host factors, such as iron, the growth rate of parasites determined by nutrient limitation and pH values consistent with environments of the vagina influence the overall protein composition of trichomonads [15]. Under steady-state conditions, dramatic qualitative and quantitative differences were observed in proteins when parasites were grown at various nutrient limitation environments and pH values found in the vagina of patients.

It is significant that sera patients with trichomonosis has antibodies to both low-iron and high-iron-induced trichomonad immunogens, showing that the parasite responds to a changing host environment [24]. This fact alone highlights the incredibly complex nature of the *T. vaginalis*—host interaction. It is clear that subpopulations of trichomonads express high- or low-iron-regulated proteins depending on the iron status of micro-environments within the vagina. It is striking that individual patients responded only to subsets of the iron-regulated proteins [24], indicating that gradients of iron concentrations within host sites affects induction of particular immunogen genes.

An extensive literature exists on the antigenic heterogeneity among *T. vaginalis* isolates. The discovery of the general property of phenotypic variation was originally defined on the basis of

surface expression and/or synthesis of repertoires of immunogens [24, 50-51]. It is now accepted that phenotypic variation by trichomonads is a response to specific parasite and environmental factors. This property now includes iron-regulated proteins [24], viral induced and repressed proteins [55], erythrocyte-binding proteins and hemolysins [7, 20], proteinases [20, 42-44, 48], adhesins [23, 34-36], among other environmentally-regulated trichomonad proteins, contribute to this parasite's overall antigenic diversity and complexity. Antibody responses among patients with trichomonosis is toward proteins that are differentially expressed depending on the specific host micro-environment(s). Therefore, populations of *T. vaginalis* organisms from fresh clinical isolates likely comprise a heterogeneous population, in which no two parasites are identical in the context of the numerous phenotypes just described. This heterogeneity among infecting parasites guarantees some parasites will successfully infect a host. The ability to generate such a heterogeneous population will further assure survival in a constantly changing host vaginal environment.

SUMMARY OVERVIEW OF THEMES

Trichomonas vaginalis is a survivor. Despite its limited genome, it is highly sophisticated in taking advantage of its unique infecting niche. While much more remains to be learned regarding virulence factors that contribute to this parasite's ability to establish a non-self limiting infection, important themes have emerged (Table 1). Of special significance is the inter-relatedness between the themes, as has been evident throughout this review. Early studies demonstrated a coating of the parasite surface with host proteins, a property that influences host recognition of a foreign agent. This work then led to identification of trichomonad receptors for specific host molecules, such as apoproteins of lipoproteins and lactoferrin. This in turn resulted in a better understanding of the nutrition acquisition systems for a parasite incapable of lipid synthesis and with a high requirement for iron. It would have been reasonable to hypothesize that the normal desquamation of target vaginal epithelial cells coupled with the normal secretions would readily rid *T. vaginalis* organisms from the vagina. This is, in part, overcome by the property of cytoadherence, mediated by four distinct surface proteins, three of which are members of multigene families. This repertoire of functional surface proteins are coordinately up-regulated in synthesis by host iron, showing that in vitro studies using batch cultures of parasites must take into account the host environment. Moreover, a long-term non-self limiting infection would eventually result in a host immune response, which would require multiple mechanisms for evasion. To this end, *T. vaginalis* organisms are uniquely qualified. Subversion of the host immune response results from the promiscuous action of the numerous trichomonad cysteine proteinases that are cytopathogenic and degrade all immunoglobulin classes. Resistance to the alternative complement pathway is due to at least one environmentally regulated proteinase that degrades C3 deposited onto parasite surfaces. The historical extensive antigenic diversity among *T. vaginalis* isolates can now be understood in the context of the discovery of phenotypic variation, as outlined above, in which no two parasites are identical and infecting populations are heterogeneous when considering numerous properties and repertoires of proteins. This complexity in environmental induction and repression of expression of trichomonad proteins has broad implications with regard to virulence and pathogenesis. Collectively, all of these properties make *T. vaginalis* a model mucosal infectious agent.

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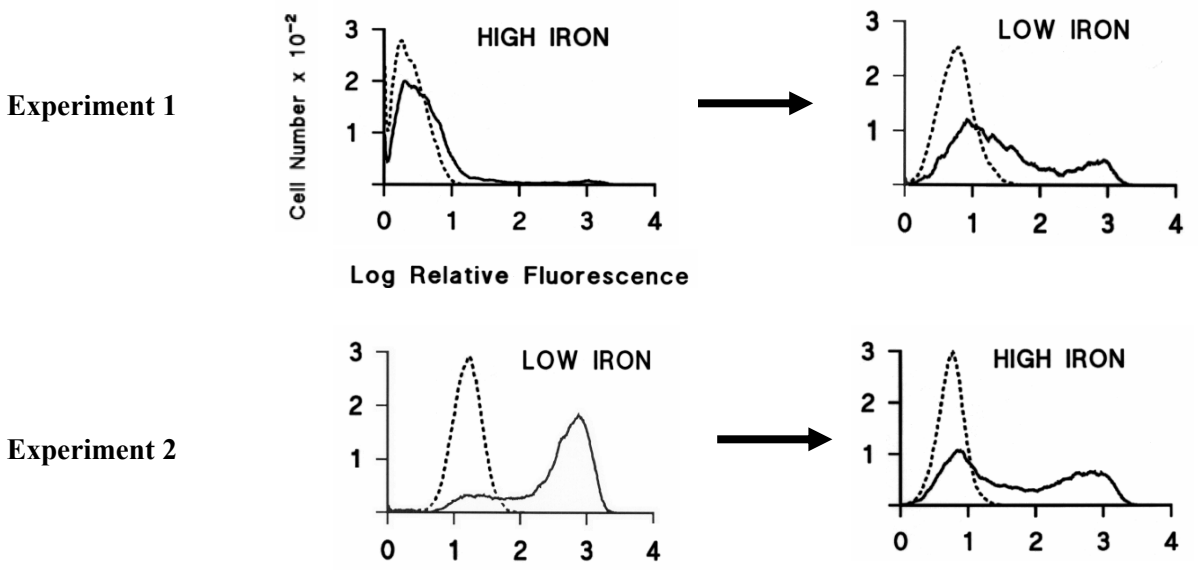

Fig 1. Flow cytofluorometry patterns using mAb C20A3 to P270 (solid lines) of live *T. vaginalis* T068-II grown overnight under high (*Experiment 1*) versus low (*Experiment 2*) iron conditions (left side patterns). An irrelevant mAb of the same IgG_{2a} isotype as C20A3 was used as a negative control (dotted lines) and is indicative of non-fluorescent organisms.

Table 1. Emerging themes and virulence factors of *T. vaginalis* contributory to pathogenesis

Themes	Virulence factor(s)
Nutrient Acquisition	Receptors for nutrient acquisition Hemagglutination and hemolysis
Cytoadherence	Receptor-ligand interactions Adhesin proteins Penetration to basement membranes
Immune Evasion	Surface coating with host proteins Shedding of immunogens Cysteine proteinase degradation of Igs and complement Phenotypic variation of immunogens
Regulation of Virulence Genes	General nutrient limitation Environmental factors (iron) Parasite factors (dsRNA virus)

Table 2. Two Naturally Occurring *T. vaginalis* Isolate Types

Isolate Type	P270 Surface Phenotype	dsRNA Virus
Type I	homogeneous, negative	−
Type II	heterogeneous, positive and negative	+