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Identification of Heme-and Hemoglobin-Binding Proteins in *Trichomonas vaginalis*

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Identification of Heme- and Hemoglobin-Binding Proteins in *Trichomonas vaginalis*

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University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of
Master of Science

Department of Biochemistry, Microbiology and Immunology
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By
Shahed Ardalan

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ABSTRACT

Trichomonas vaginalis is the cause of human trichomoniasis. Although acquisition of iron by binding to host hemoglobin through distinct receptor(s) has been described in this parasite, no specific hemoglobin-binding site has been reported.

Our hypotheses were: (1) hemolytic activity of *T. vaginalis* isolates correlates with the virulence, and (2) *T. vaginalis* binds hemoglobin through a specific surface receptor.

The in vitro hemolytic activities of four *T. vaginalis* strains were examined. No correlation between hemolytic activity and virulence in different isolates was found.

Using hemoglobin-affinity chromatography, two protein bands of approximately 48 and 63 kDa were detected, the former binds preferentially to heme. Mass spectral analysis indicated that the 48- and 63-kDa proteins had significant homology with the subunits of two *T. vaginalis* adhesins: AP51 and AP65, respectively.

This study confirms the existence of multifunctional proteins in *T. vaginalis*, which enable the parasite to survive in a constantly changing environment like human vagina.

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LIST OF ABBREVIATIONS

2,2-DP	2,2-dipyridyl
AP120	120 kDa surface protein
AP23	Adhesion Protein with molecular mass of 23 kDa
AP33	Adhesion Protein with molecular mass of 33 kDa
AP51	Adhesion Protein with molecular mass of 51 kDa
AP65	Adhesion Protein with molecular mass of 65 kDa
AS	Antisense
AT	Agglutination Test
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid assay
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CDF	Cell-Detaching Factor
CPE	Cytopathic Effects
CPs	Cysteine Proteinases
DFA	Direct Fluorescence Antibody assay
DFO	Deferoxamine
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
ds	double-stranded
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked ImmunoSorbent Assay
FBS	heat-inactivated Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
HABA (dye)	4'-hydroxyazobenzene-2-carboxylic acid
HBSS	Hank's Balanced Salt Solution
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
hVECs	human Vaginal Epithelial Cells
IFA	Indirect Fluorescent Antibody assay

Ig	Immunoglobulins
kDa	kilodalton
LBP	Laminin-Binding Protein
LF	Lactoferrin
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
NCBI	National Center for Biotechnology Information
PBS	Phosphate Buffered Saline
PBS-T	PBS and 0.1% Tween 20
PCR	Polymerase Chain Reaction
PFO	Pyruvate:Ferredoxin Oxidoreductase
PFO	Pyruvate:Ferrodoxin Oxidoreductase
PLA	PhosphoLipase A
PROM	Premature Rupture Of Membranes
PVDF	Polyvinylidene Fluoride (PVDF) membrane
RBCs	Red Blood Cells
RNA	Ribonucleic acid
S	Sense
SAEs	Surface-Associated Enzymes
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SLPI	Secretory Leukocyte Protease Inhibitor
STD	Sexually Transmitted Disease
TCA	Trichloroacetic Acid
TF	Transferrin
TLCK	<i>N</i> - α - <i>p</i> -tosyl-L-lysine Chloromethyl Ketone
TV44	a 44-kDa protein on <i>Trichomonas vaginalis</i> surface
TVVs	<i>Trichomonas vaginalis</i> viruses
TYI-S-33	Diamond's Trypticase-Yeast extract-Iron medium
TYM	Trypticase-Yeast extract-Malto
WHO	World Health Organization
α -SCS	α - Succinyl Coenzyme A Synthetase
β -SCS	β - Succinyl Coenzyme A Synthetase

CHAPTER ONE: INTRODUCTION

1.1. General Information

Trichomonas vaginalis, a parasitic protozoan, is the main cause of human trichomoniasis, which is the most common non-viral sexually transmitted disease (STD) worldwide. *T. vaginalis* belongs to the family Trichomonadidae, is characterized by having four free anterior flagella (the fifth flagellum forms the outer edge of the undulating membrane), and a rigid structure called axostyle, which extends through the body from the anterior to the posterior end (Schwebke and Burgess, 2004). Different shapes and sizes of this parasite have been observed depending upon the environmental conditions. On average, the organism is 10µm long and 7µm wide, and has an amoeboid shape when adhered to vaginal epithelial cells (Arroyo et al., 1993). In axenic culture and under favourable growth conditions, the trichomonads can look pear shaped or oval. Under unfavourable growth conditions, the cells tend to round up and internalize their flagella and form a cyst-like shape. Since these “pseudocysts” are unable to regain the oval, flagellated form when re-established in normal growth conditions, they are considered a degenerate form of this parasite (Honigberg and Brugerolle, 1990). *T. vaginalis* has a nucleus, surrounded by a porous membrane, located at the anterior part of the cell (Petrin et al., 1998).

Unlike most eukaryotes, this anaerobic protozoan does not have typical mitochondria. Instead, there are specialized organelles called hydrogenosomes, which are involved in energy metabolism (Horner et al., 2000). Pyruvate produced by classical glycolysis in the cytosol is further metabolized in hydrogenosome to acetate, CO₂ and hydrogen (Müller, 1993). The origin of hydrogenosome is not clear, but it is hypothesized that it has a common ancestry with mitochondria. Despite some common features in protein import and ATP synthesis, hydrogenosomes and mitochondria have major

differences, with the hydrogenosomes lacking cytochromes, respiratory chain enzymes, oxidative phosphorylation, and DNA (de Andrade Rosa et al., 2006).

Like some other protozoan organisms (*Giardia*, *Leishmania*, and *Eimeria*), some clinical *T. vaginalis* strains are infected with a double-stranded (ds) RNA virus. Although *T. vaginalis* viruses (TVVs) were described as homogenous populations of icosahedral viruses with isomeric symmetry, Benchimol et al. (2002) showed that the virus population is highly heterogeneous. They observed viruses in different sizes, ranging from 33 to 200 nm, and various shapes from filamentous to cylindrical or spherical. It has been suggested that *T. vaginalis* might act as a reservoir for different dsRNA viruses (Benchimol et al., 2002). It has been shown that trichomonads infected with virus, express a highly immunogenic surface protein called P270 (Wang et al., 1987). It has also been suggested that the presence of this dsRNA may be related to susceptibility of *T. vaginalis* to metronidazole, the drug used for treatment of *T. vaginalis* infection (Wang and Wang, 1985). However, the exact role of these TVVs in the pathogenesis and immunogenesis of *T. vaginalis* is still unclear.

1.2. Life Cycle

The life cycle of *T. vaginalis* is still unclear. Humans are the only known host for this flagellated protozoan. It seems that this parasite has a simple life cycle. There is no cyst formation, thus transmission occurs via sexual transmission of the trophozoite stage (Honigberg and Brugerolle, 1990). The main target is the mucosal surfaces of the urogenital tract where trophozoites attach, replicate, reach a population density enough to overcome the host defence mechanisms, and establish infection (Schwebke and Burgess, 2004).

T. vaginalis presents one of the most primitive kinds of mitosis called “closed” mitosis or pleuromitosis, in which the extranuclear microtubular spindle does not contact the chromosomes directly and nuclear membrane remains intact (Heath, 1980; Zuo et al., 1999).

1.3. Experimental models

To provide a better understanding of the mechanisms responsible for pathogenesis, virulence factors, and host response of *T. vaginalis*, this parasite has been studied in a variety of animal models including monkeys, hamsters, rats, dogs, and mice. The mouse is the most popular animal model used in *T. vaginalis* studies (Petrin et al., 1998). However, there are major differences between the human female genital tract and that of mice. For successful establishment of infection, mice are pre-inoculated with *Lactobacillus acidophilus* (a normal constituent of human vagina) and pre-treated with estrogens (McGrory and Garber, 1992).

More information about host-parasite relationships of *T. vaginalis* can be acquired by studying *Tritrichomonas foetus*, a trichomonad species closely related to *T. vaginalis*, which infects the genital tract of cattle and causes fetal loss. Since both trichomonads share many virulence factors, the bovine model can also provide useful information about trichomoniasis in human (Corbeil, 1995).

1.4. Epidemiology

Being the only natural host for *T. vaginalis*, more than 170 million women around the world are infected by this parasite annually (WHO, 2001). In the United States, approximately 5 million new cases of trichomoniasis occur each year (Soper, 2004), among them African-American populations are at higher risk compared to any other ethnic or racial group (Lehker and Alderete, 2000). People in developing countries and those with low socio-

economic status, have higher prevalence rate of trichomoniasis (Mabey et al., 2006). Older age, pregnancy, drug use, and prostitution are all factors that could increase the incidence of trichomoniasis (Swygard et al., 2004). Trichomoniasis is commonly associated with other STDs such as gonorrhea. The majority of women infected with *T. vaginalis* also have bacterial vaginosis. Unlike other STDs, there is no higher incidence of trichomoniasis among adolescents and young adults (Schwebke and Burgess, 2004). It could be due to reproductive hormone levels, longer duration of infectiousness, or to host-parasite relationships (Soper, 2004; Swygard et al., 2004). Since trichomoniasis rates are higher in sexually active women of all age groups, this STD could be considered as a marker for high-risk sexual behavior (Schwebke and Burgess, 2004).

Although trichomoniasis is a common sexually transmitted infection among women, the prevalence and spectrum of this disease is not well characterized in men (Seña et al., 2007). It is probably because trichomoniasis in men is mostly asymptomatic and it is believed that the infection is often self-limited. Diagnostic methods are not sensitive enough to detect this parasite in men (Soper, 2004). Asymptomatic male carriage is the source of infection in women.

1.5. Clinical presentation

Trichomoniasis symptoms range from asymptomatic to severe. In women, vaginitis and increased vaginal discharge are the most common complaints. The discharge is often profuse with itching and burning. In the severe form of infection, the discharge is mainly frothy and mucopurulent with a green or yellow colour. In 2% of patients, the vaginal and cervical mucosa look diffusely hyperaemic with bright-red punctate lesions, termed “strawberry appearance”. The symptoms are cyclic and often get worse during menstruation

(Petrin et al., 1998). Since *T. vaginalis* produces amine during growth, the vaginal pH is elevated (greater than 4.5). Changes in normal vaginal ecology may subsequently result in bacterial vaginosis (Soper, 2004; Seña et al., 2007). Several studies demonstrated that infection with *T. vaginalis* is correlated with low birth weight infants, premature rupture of membranes (PROM), and premature delivery. Similar outcome has been found in bovine trichomoniasis, caused by *Tritrichomonas foetus*, which results in abortion in cattle. The linkage between trichomoniasis and adverse outcomes of pregnancy is still unknown. One hypothesis is that infection with *T. vaginalis* elevates levels of proteolytic enzymes and cytokines within vaginal or cervical fluids, and the presence of cytokines within the amniotic fluid could result in chorioamnionitis and pre-term birth (Schwebke and Burgess, 2004). Finally, women infected with *T. vaginalis* might be at an increased risk of developing cervical cancer (Soper, 2004).

In contrast to women, trichomoniasis in men is largely asymptomatic and they are mainly considered carriers for this disease (Petrin et al., 1998). Non-gonococcal, non-chlamydial urethritis is the most common complaint of men (Hobbs et al., 1999). In addition to urethritis, untreated trichomoniasis may result in epididymitis and chronic prostatitis (Seña et al., 2007).

Studies have shown that infection with *T. vaginalis* can cause infertility in both men and women. One reason is that trichomonads could act as a vector, carrying other pathogens attached to its surface into the fallopian tubes, thus increasing the risk of tubal infertility. In men, trichomoniasis causes significant decrease in both sperm motility and viability, resulting in infertility (Soper, 2004).

Trichomoniasis has been associated with HIV risk amplification among both men and women (Soper, 2004). Several studies have demonstrated that the rate of HIV-1 infection is

higher in women with active urogenital trichomoniasis when compared to women with normal vaginal flora (Rendón-Maldonado et al., 2003). It has been suggested that symptomatic inflammatory trichomoniasis in men may increase HIV excretion in semen (Soper, 2004). The increased HIV infectivity among HIV/*T. vaginalis* co-infected individuals may be related to: (i) the focal mucosal lesions produced by trichomonads cause macro- or microscopic break in mucosal barriers, which provide a direct viral access to the bloodstream through these open lesions; (ii) the vaginal discharge in women containing massive numbers of CD4+ T lymphocytes, the main target cell for HIV; (iii) local inflammation caused by *T. vaginalis* might increase HIV shedding; (iv) *T. vaginalis* is able to degrade secretory leukocyte protease inhibitor, a product known to block HIV cell attachment, which may also facilitate HIV transmission; (v) *T. vaginalis* itself, could serve as a vehicle for the transmission of HIV particles (Sorvillo et al., 2001; Rendón-Maldonado et al., 2003; Schwebke and Burgess, 2004). Since trichomoniasis is the most common non-viral STD among HIV patients, even a small decrease in its prevalence could result in concomitant reductions in the spread of HIV. Therefore, efforts to prevent and treat infections with *T. vaginalis* thoroughly might be able to successfully and inexpensively reduce the incidence of HIV transmission (Soper, 2004).

1.6. Diagnosis

The clinical presentation of trichomoniasis is neither sensitive nor specific enough to be a reliable diagnostic tool. When the diagnosis is based solely on clinical symptoms, 88% of women infected with *T. vaginalis* will not be identified and 29% of women will have a false positive diagnosis (Fouts and Kraus, 1980). Moreover, since most patients with trichomonal infections are asymptomatic or mildly symptomatic, appropriate laboratory

testing is usually required to establish an accurate diagnosis (Petrin et al., 1998). Several diagnostic methods are used to detect *T. vaginalis* in men and women (Soper, 2004). Wet mount microscopic examination of suitable clinical specimens including urine, vaginal fluid, and semen, is the most commonly used diagnostic method for the detection of *T. vaginalis*. Characteristic motile trichomonads are readily seen. This test must be performed within 10 to 20 min after the sample is collected as motility diminishes with time. Although this test is quick and inexpensive, its sensitivity is limited, ranging from 60% to 70% compared with culture method, and requires microscopic expertise (Schwebke and Burgess, 2004; Mabey, et al., 2006).

Culture in Diamond's medium, considered as the "gold standard" for diagnosis of *T. vaginalis* infection, has a significant degree of sensitivity and specificity, and will successfully identify more than 95% of infections (Soper, 2004). The advantage of this test is that as few as 300 to 500 trichomonads per milliliter of inoculum is needed to initiate growth in culture. However, there are some disadvantages for culture diagnosis. This test is time consuming, an incubation period of 2 to 5 days is usually needed to make a definitive diagnosis, and during this time infected patients may infect other people. Since culture media are not readily available and are more expensive than wet mounts, this method is mainly used for research purposes. (Schwebke and Burgess, 2004; Soper, 2004). The InPouch TV culture system is a commercially available medium. It consists of a two-chambered bag and allows performing both culture and microscopic examination of the specimen (Patel et al., 2000). Studies have demonstrated that this system is as sensitive as the Diamond's medium culture technique in recovering *T. vaginalis* isolates and could be considered as a suitable substitution for the culture method (Draper et al., 1993). Papanicolaou (Pap) smear can be used as a screening tool for trichomoniasis with a sensitivity of about 61% (Swygard, et al., 2004). Women

whose Pap test results are positive for *T. vaginalis* are recommended to undergo confirmatory testing (Aslan et al., 2005).

Antibody-based diagnostic techniques to identify trichomoniasis include enzyme-linked immunosorbent assay (ELISA), direct fluorescence antibody assay (DFA), enzyme immunoassay (EIA), indirect fluorescent antibody (IFA) assay, and agglutination test (AT) have sensitivities in the range of 70% to 90% (Krieger et al., 1988; Patel et al., 2000).

More recently, newer diagnostic methods like polymerase chain reaction (PCR) have been described for the detection of *T. vaginalis* DNA in clinical samples with sensitivity between 85% to 100% (Schwebke and Burgess, 2004; Mabey et al. 2006). Although PCR-based tests for detecting trichomoniasis in women does not have much of an advantage compared to the other methods, it appears to be more sensitive in the diagnosis of this infection in men (Schwebke and Burgess, 2004). PCR analysis of urine specimens is significantly more sensitive than culture for detection of trichomoniasis in men (Schwebke and Lawing, 2002). However, due to its costliness, it has not been applied in routine diagnosis of *T. vaginalis* infection, but it could be used as an alternative laboratory diagnosis.

Studies have shown that there is an advantage to using combined diagnostic methods over one test alone (Radonjic et al., 2006). Combining diagnostic methods can facilitate the detection of trichomoniasis, which subsequently could result in better control and treatment of *T. vaginalis* infection in women and men.

1.7. Treatment

T. vaginalis was discovered in 1836 and has been known to cause vaginitis since 1916. However, it was not until 1957 that an effective cure, metronidazole, was discovered (Upcroft and Upcroft, 2001). Metronidazole, as well as several other 5-nitroimidazole

derivatives, strongly inhibits biological activity in anaerobic protozoa and anaerobic bacteria (Müller and Lindmark, 1976). Metronidazole enters the cell through diffusion and is then activated in the hydrogenosomes of *T. vaginalis*. There, the nitro group of the drug is reduced anaerobically by pyruvate-ferredoxin oxidoreductase, which results in cytotoxic nitro radical-ion intermediates that break the DNA strands whereupon cell division and motility arrest within one hour, and cell death occurs within 8 hours (Nielsen, 1976).

The standard treatment for trichomoniasis is 250 mg of metronidazole given orally three times a day for 7 days, 500 mg twice a day for 7 days, or in a single 2-g dose (Cudmore et al., 2004). Both symptomatic and asymptomatic patients and their sexual contacts should be treated to prevent recurrent infection (Heine and McGregor, 1993). Pregnant women diagnosed with trichomoniasis may be treated with the 2-g single dose of metronidazole, particularly in the last two trimesters of pregnancy (Schwebke and Burgess, 2004).

Side effects of metronidazole are usually mild and include nausea, vomiting, headache, insomnia, dizziness, drowsiness, rash, dry mouth, and metallic taste. However, some patients have severe reactions to metronidazole like eosinophilia, leukopenia, and palpitation. These side effects are temporary and recede after therapy ends (Cudmore et al., 2004).

Reports of metronidazole-resistant strains of *T. vaginalis* have been recently increased (Ryu and Min, 2006). Developing drug resistance in trichomonads shows how this parasitic protozoan is able to adjust its metabolic pathways in response to the pressure of an unfavourable environment. Although metronidazole resistance is uncommon, its incidence is on the rise. This is a concern, especially since this drug is the only approved treatment for trichomoniasis in the United States (Kulda, 1999). Treatment failure can result in significant clinical morbidity and increase the risk of disease spread.

Tinidazole is a 5-nitroimidazole compound that is chemically related to metronidazole and has widely used outside of the United States for the treatment of trichomoniasis. A 2-g oral dose of tinidazole has an overall clinical efficacy equal to metronidazole. Tinidazole may be an option for treating patients with metronidazole-resistance trichomoniasis (Schwebke and Burgess, 2004; Soper, 2004).

1.8. Pathogenesis

As previously mentioned, there is a high incidence of *T. vaginalis* especially among women around the world, which causes important clinical problems including obstetric complications. However, little is known about the host-parasite relationship, and the pathogenesis of trichomoniasis is not completely understood (Carvalho et al. 2005). Its broad spectrum of clinical symptoms cannot be easily explained by a single pathogenic mechanism.

Trichomonads mainly parasitize the vaginal epithelium, which due to the menstrual cycle, is a particularly complex site for the establishment of an infection. *T. vaginalis* has developed several mechanisms in order to survive and maintain the infection in such a changeable environment (Petrin et al., 1998; Lehtker and Alderete, 2000). During menstruation, *T. vaginalis* habitat undergoes significant changes: the vaginal pH changes, and there are massive amount of serum proteins, erythrocytes and host macromolecules in the menstrual flow. It is at this time that the alternative complement pathway encounters trichomonads. Although it has been shown that menstrual blood complement is trichomonocidal, surprisingly some authors have reported that symptoms of trichomoniasis worsen during and/or shortly after menstruation (Lehtker et al., 1991; Ryu et al, 2001). Apparently *T. vaginalis* is able to regulate the expression of virulence factors on its surface to escape complement lysis (Alderete et al., 1995b). This finding indicated that *T. vaginalis*,

through its signal transduction pathways, can successfully respond to different external stimuli, and establish infection in an adverse host environment (Lehker and Alderte, 2000).

Contact-dependent and contact-independent mechanisms are both important in the pathogenesis of *T. vaginalis*. Cytoadherence, hemolysis, proteinase production, cell-detaching factor (CDF), and evasion of host immune system all have key roles in pathogenesis (Bhatt et al., 1996). These mechanisms are discussed in more detail in the following sections.

1.8.1. Adherence and Adhesins

Many mechanisms are thought to be involved in the early stage of establishing a vaginal infection. One of the critical steps in pathogenesis of the parasite is cytoadherence (Petrin et al., 1998). Trichomonads adhere to urogenital epithelial cells; induce cell cytotoxicity, which subsequently results in damaging the epithelium (Furtado and Benchimol, 1998). Adhesion of trichomonads to the epithelial cells is a receptor-ligand-type interaction, and is time, temperature and pH dependent (Alderete et al. 1995a). Most studies on cytoadherence in *T.vaginalis* have been carried out in an in vitro model using immortalized human vaginal epithelial cells (hVECs), or HeLa cells (Kucknoor et al. 2005b).

Upon binding to the host cells, trichomonads undergo significant changes in their morphology. The ability of *T.vaginalis* to transform from oval/pear-shaped to an amoeboid form, which is considered as a virulence trait, requires the presence of a complex cytoskeletal structure. Alpha-actinin, an actin-binding protein that participates in rearrangement of actin fibers, has been described in *T. vaginalis*. This finding suggests that alpha-actinin may play an important role in mediating morphological changes in *T. vaginalis* (Addis et al., 1998). In pear-shaped trichomonads, alpha-actinin is diffused throughout the

entire cytoplasm (Fiori, et al. 1999). It has been reported recently that a 44-kDa protein (TV44) on *T.vaginalis* surface might be responsible for maintenance of the ovoid form in non-adherent parasites. Surface expression and amount of TV44 is down-regulated upon contact with vaginal epithelial cells (Mundodi et al., 2006). When trichomonads adhere to the target cells, redistribution in alpha-actinin occurs, transforming the parasite to an amoeboid form (Arroyo et al., 1993). In the amoeboid form of *T.vaginalis*, alpha-actinin is mainly localized in the peripheral regions, lining the pseudopodal extensions of the parasite. This morphological transformation is fundamental for pathogenicity (Addis et al., 1998). Studies have shown that anti-cytoskeletal drugs can interfere with the cytopathic effects of the parasite (Juliano et al., 1987).

Binding between the parasite and the target cell forms special spaces between them, which allow the parasite to alter the condition of the microenvironment for its own benefit (Fiori, et al. 1999). Following recognition and binding of *T.vaginalis* to the epithelial cells, another signal cascade begins. It is different from the first signal, which is responsible for transformation, and is involved in stimulating parasite to increase the synthesis of adhesion proteins (Alderete et al. 1995a). Four adhesion proteins (adhesins) with molecular masses of 65kDa (AP65), 51kDa (AP51), 33kDa (AP33), and 23kDa (AP23) have been identified in all the examined isolates of *T.vaginalis* (Arroyo et al. 1992). The level of trichomonad adherence to vaginal epithelial cells is equally related to the amount of these four adhesins (Alderete et al., 1998). Using antibody to each adhesin, these proteins were detected on the surface of live trichomonads. Pretreatment of epithelial cells with purified adhesins could inhibit cytoadherence by *T.vaginalis* (Alderete et al. 1995a). The synthesis of these four adhesins is co-ordinately upregulated by binding to epithelial cells and by the presence of iron (Lehker and Alderete, 2000).

Recent studies have shown that three of *T.vaginalis* adhesins AP65, AP51, and AP33 are each encoded by a multiple-gene family (Engbring and Alderete, 1998). Sequence analysis at both the nucleotide and amino acid level, reveal that these three adhesins have identity with the hydrogenosome enzymes decarboxylating malic enzyme, β -succinyl coenzyme A synthetase (β -SCS), and α - succinyl coenzyme A synthetase (α -SCS), respectively (Garcia et al. 2005).

AP65 has a prominent role in the adhesion of *T.vaginalis* to hVECs (Kucknoor et al., 2005a). Recently, the expression of *ap65* was silenced by antisense RNA gene silencing. It was demonstrated that synthesis of AP65 decreased in AS transfectants when compared to wild type or S transfectants, and consequently the amount of AP65 binding to epithelial cells decreased in AS transfected parasites. However, decreased amount of AP65 in *T.vaginalis* did not adversely affect its growth rate and energy metabolism (Mundodi et al., 2004).

It is believed that cytoadherence in *T.vaginalis* occurs in a ligand-receptor fashion, and adhesins bind to specific sites found on the surface of the host cells (Arroyo et al., 1992). However, no putative receptors on the host cells have been identified yet. It has been suggested that rather than specific receptors on the target cells, an affinity for the other cell structures like membrane could participate in the parasite cytoadherence (Addis et al., 2000).

Recently, Arroyo's group identified a 120kDa surface protein (AP120), which is involved in *T. vaginalis* cytoadherence. AP120, like the other three adhesins, has homology with a hydrogenosomal enzyme pyruvate:ferredoxin oxidoreductase (PFO). Synthesis of AP120, like other adhesins, is regulated at transcriptional level by iron concentration and cellular contact. Unlike other adhesins, a 130kDa surface protein in HeLa cells has been identified as a putative receptor for the AP120 (Moreno-Brito et al., 2005).

No similar adhesion proteins have been detected in non-pathogenic trichomonads like *Trichomonas tenax* (Alderete and Garza, 1988). However, adherence is not directly correlated with virulence, since different *T. vaginalis* isolates from symptomatic patients have differing binding properties to host cells (Krieger et al., 1990).

Overall, the cytoadherence of *T. vaginalis* to host cells is a complex and multifactorial mechanism, which is crucial for colonization and persistence of the parasite. The presence of adhesins on the *T. vaginalis* surface is necessary but not sufficient for cytoadherence (Gilbert et al., 2000). Along with adhesins, cysteine proteinases are additional requirements for successful binding of pathogen to its target cells (Alderete et al., 1995a).

1.8.2. Proteinases

T. vaginalis has multiple forms of proteinases, most of which are secreted during axenic growth (Lockwood et al., 1987). However, trichomonad proteinases have been detected in vaginal secretions of infected women showing that this could occur in vivo as well (Lehker and Sweeney, 1999). Proteinases released by *T. vaginalis* are mainly secreted from, or via, lysosomes (Scott et al., 1995). These proteinases can degrade a variety of host substrates such as hemoglobin, laminin, and immunoglobulins, and are related to nutrient acquisition, along with virulence properties, such as immune evasion, cytoadherence, hemolysis, and cytotoxicity (Lehker and Sweeney, 1999; Alvarez-Sánchez et al., 2000).

Early studies revealed that at least 11 different cysteine proteinases are present in *T. vaginalis*. Up to 23 distinct proteinase activities in several *T. vaginalis* isolates have been reported by using two-dimensional substrate-gel electrophoresis. These proteinases had relative molecular masses between 23 and 110 kDa (Neale and Alderete, 1990). The exact function of many trichomonad cysteine proteinases (CPs) is still unclear (Provenzano and

Alderete, 1995). It has been shown that CP activity is necessary for parasite adhesion to HeLa cell monolayers (López et al., 2000). Pretreatment of *T. vaginalis* with *N*- α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK), which is a cysteine proteinase inhibitor, greatly decreases the ability of trichomonads to recognize and bind to epithelial cells, indicating that proteinase activity on trichomonad surface is required for cytoadherence (Arroyo and Alderete, 1989). A 30-kDa proteinase in *T. vaginalis* enables the parasite to degrade spectrin, the major cytoskeleton protein in cells, causing epithelial cytoskeletal disruption (Fiori et al., 1997). Using mass spectrometric analysis, Sommer et al. (2005) identified several CPs secreted from *T. vaginalis* that induce apoptosis in human vaginal epithelial cells. The ability of the isolated CPs to induce hVEC apoptosis is inhibited by a specific CP inhibitor, E-64.

Two extracellular proteinases with molecular masses of 60 and 30 kDa have been detected in cell-free filtrates of *T. vaginalis*. The finding that these proteinases are active over a broad acid-neutral pH range suggests that they could have a key role in the establishment of the infection (Garber and Lemchuk-Favel, 1989).

As mentioned earlier, the risk of HIV transmission and other ulcerative STDs increase in the presence of trichomoniasis. Secretory leukocyte protease inhibitor (SLPI) is an abundant serine protease inhibitor found on many mucosal surfaces (Sajid and McKerrow, 2002). It has several functional effects on inflammatory cells, and also has an antimicrobial property. It has been shown that SLPI has an important role in natural defense against HIV. In women infected with trichomoniasis, the level of SLPI in vaginal fluids is lower due to the proteinases produced by trichomonads. These cysteine proteinases are able to degrade SLPI into smaller peptides and destroy its functional activity; therefore, increasing the risk of HIV acquisition in these women (Draper et al. 1998).

Several *T. vaginalis* proteinases have important roles in immune evasion mechanisms, including degradation of human immunoglobulins (Ig), and resistance to the alternative complement pathway (Alderete et al. 1995b). All *T. vaginalis* isolates have numerous immunoglobulin-degrading CPs with different molecular masses and the ability to degrade human IgG, IgM, and IgA (Provenzano and Alderete, 1995; Min et al., 1997).

It has been suggested that *T. vaginalis* CPs could be used as diagnostic markers, or therapeutic targets (Sommer et al., 2005). Further research on these CPs could provide new insights into pathogenicity, diagnosis, and treatment of trichomoniasis.

1.8.3. Hemolysis

In order to survive, *T. vaginalis* has evolved and adjusted to the vaginal environment, a relatively hostile environment with scarce nutrients (Rendón-Maldonado et al., 1998). Trichomonads are unable to synthesize their essential nutrients such as lipids (Peterson and Alderete, 1984). However, they are able to lyse erythrocytes, which represent an important source of iron and lipids (Lehker et al., 1990). The erythrocyte membranes are rich in cholesterol, which can be acquired by the parasite. Iron, another key nutrient for *T. vaginalis*, is acquired from hemoglobin following red blood cell hemolysis. Exacerbation of the clinical symptoms of trichomoniasis during or immediately after menstruation may be related to the increased number of erythrocytes in the vagina during menses (Dailey et al., 1990). In vitro isolates of *T. vaginalis* have the ability to lyse all human blood groups, as well as, rabbit, rat, chicken, horse, bovine, and sheep erythrocytes (de Carli et al., 1998).

Krieger et al. (1983) demonstrated that *T. vaginalis* has beta-hemolytic activity, and adhesion of trichomonads to red blood cells (RBCs) is a prerequisite for this activity. They suggested that the hemolytic activity might be related to the virulence.

Five *T. vaginalis* adhesion proteins that bind to the RBC membranes were detected by Western blot, using a rabbit anti- *T. vaginalis* antiserum as a probe. The molecular masses of these adhesion molecules range from 140 kDa to 33 kDa. Three of these adhesins are the same proteins associated with cytoadherence (AP65, AP51, and AP33), but the other two (140 and 42 kDa) are specific for erythrocyte binding (Fiori et al., 1993). It was also shown that trichomonal proteins are present on the surface of RBC after one-hour incubation with *T. vaginalis*. Although these RBCs are still intact, they are extremely fragile, and are completely lysed within one hour (Fiori et al., 1993). Inhibitors of *T. vaginalis* cysteine proteinases greatly reduce or abolish hemolytic activity, indicating that one or more of the parasite CPs is involved in hemolysis (Dailey et al., 1990). On the basis of these findings, the hemolytic activity of *T. vaginalis* could be divided into three distinct steps: (1) a specific ligand-receptor interaction allows *T. vaginalis* to recognize and strongly bind to RBC; (2) toxic molecules (probably CPs) are released from the parasite, forming pores in the erythrocyte membrane; and (3) *T. vaginalis* detaches itself from the RBC, and hemolysis occurs (Fiori et al., 1993; Rosset et al., 2002).

Hemolytic activity in *T. vaginalis* is temperature- dependent, and is maximal at 37°C. The activity decreases at lowering temperature, and is completely inhibited at 4°C. It is also pH-dependent. Higher levels of hemolysis are observed in a pH range of 5-6, and is reduced by more than 70% as the pH becomes higher than 7.0 (Dailey et al., 1990). The pore-forming protein function is optimal at a pH lower than 6.5. Pore-forming proteins are able to insert themselves into the membrane of erythrocytes and form transmembrane channels, which result in cell lysis (Fiori et al., 1993).

As discussed earlier, microenvironments form between trichomonads and target cells after adherence provide optimal condition for hemolysis. These isolated intercellular spaces

allow trichomonads to control the local pH environment, and concentrate their pore-forming proteins (Fiori et al., 1999).

Vargas-Villarreal et al. (2003) showed that *T. vaginalis*, like other parasitic protozoa, has phospholipase A (PLA) on its plasma membrane, and hemolytic activity is at least partially due to this enzyme. Most PLAs lyse erythrocytes by directly hydrolyzing membrane phospholipids (Vargas-Villarreal et al., 2005).

It has been suggested that hemolytic activity is a contact-dependent phenomenon and microscopic studies have shown a significant correlation between hemolysis and adherence of the erythrocytes to the surface of *T. vaginalis* (Potamianos et al., 1992). However, Fiori et al. (1996) reported that *T. vaginalis* can release proteins that are able to lyse erythrocytes without any direct contact between the parasite and its target cell. This study confirmed the findings of Pindak et al. (1993). They had demonstrated that cell-free filtrates of *T. vaginalis* have the same degree of hemolytic activity as when the intact organism is incubated with erythrocytes. A pH-dependent lytic protein with a molecular mass higher than 30 kDa could be responsible for contact-independent hemolysis (Rosset et al., 2002).

Investigation of the hemolytic activity of *T. vaginalis* could provide a better understanding of cytopathic mechanisms used by this parasite in order to successfully survive in vivo (Dailey et al., 1990).

1.8.4. Cell-Detaching Factor (CDF)

Contact-dependent cytotoxicity is not the only mechanism that is important in the pathogenesis of *T. vaginalis*. Contact-independent mechanisms also have significant roles in the pathogenesis of this parasite (Lubick and Burgess, 2004). In 1943, Hogue first reported that cell-free filtrates of *T. vaginalis* had similar cytopathic effects on cell cultures to those of

the organism itself, and hypothesized that the pathogenesis observed was directly due to secreted toxins from *T. vaginalis*. Subsequently some soluble cytotoxin from cell-free, parasite-derived filtrates with cyto-pathogenic effects on cultured cells has been reported (Garber et al., 1989; Pindak et al., 1993).

In addition to the identification of a number of tissue culture cell types suitable for the propagation of *T. vaginalis*, Pindak et al. (1986) demonstrated the presence of a substance, which was a likely cause of the cytopathic effects (CPE) mentioned by earlier investigators, and tentatively called it cell-detaching factor or CDF. They showed that the CDF is a heat and acid labile factor, and its production is not limited to any particular strain of *T. vaginalis* or to its cultivation on any specific cell culture type.

Garber et al (1989) showed that cell-free filtrate of all tested *T. vaginalis* isolates produced a monolayer detaching effect. The detachment of the cell monolayers in vitro was thought to be analogous to the sloughing of vaginal epithelial cells seen in the vaginal mucosa during acute infections. They isolated and characterized the CDF, which is a 200-kDa glycoprotein, and is heat and acid labile; pH lower than 5.0 will inactivate the CDF on the monolayer. Production level of the CDF is associated with the severity of the clinical symptoms of trichomonal vaginitis. Therefore, CDF could be considered as a virulence marker for this disease (Garber and Lemchuk-Favel, 1990).

The CDF production is likely influenced by the concentration of estrogen in the vagina. In vitro studies have been shown that the production of CDF by *T. vaginalis* significantly decreases in the presence of β -estradiol. It is possible that the levels of estrogen in the vagina could influence the degree of symptoms associated with trichomoniasis, as a result of the effect of estrogen on CDF production (Garber et al., 1991). The CDF is immunogenic, and the detaching activity is inactivated by human sera reactive to

T. vaginalis. Thus, it could also be considered as a useful tool in diagnosis of trichomoniasis (Garber et al., 1989).

1.8.5. Immune System Evasion

Natural and acquired immune responses are evident in patients with trichomoniasis, but this parasite, like many other parasites, has evolved a variety of strategies for surviving in the host (Bloom, 1979; Lehter and Alderete, 2000). The observation that *T. vaginalis* is killed by polymorphonuclear neutrophils in the presence of fresh human serum, suggested that *T. vaginalis* activates the alternative complement pathway, and this activation can lead to the parasite lysis. However, as a strategic tactic, *T. vaginalis* is able to avoid lysis by complement, and overcome the human immune system (Gillin and Sher, 1981). It appears that some CPs on the surface of this parasite can degrade the C3 portion of the complement, which allows the organism to evade complement-mediated destruction (Alderete et al., 1995b).

Phenotypic variation, the capability of *T. vaginalis* to alternate between surface versus non-surface expression of specific high molecular weight immunogens, is another mechanism of host immune evasion (Alderete et al., 1987; Musatovova and Alderete, 1998). Trichomonads that undergo phase variation during growth and multiplication may be capable of evading humoral immune mechanisms in their host (Alderete and Kasmala, 1986).

In addition to these mechanisms, *T. vaginalis* has many other ways of evading the immune system. The numerous CPs synthesized by *T. vaginalis* degrade all subclasses of immunoglobulins, which allows the organism to survive an antibody response. As mentioned earlier, trichomonad proteinases are able to degrade SLPI, and render this protective inhibitor non-functional (Lehter and Alderete, 2000).

T. vaginalis possesses the ability to loosely bind host plasma proteins. Coating of parasite surfaces with host molecules does not allow the host's immune system to recognize the parasite as foreign. Thus, immune factors in the vagina are effectively neutralized, allowing trichomonads to dwell in an otherwise hostile environment (Peterson and Alderete, 1982).

Lastly, *T. vaginalis* is a phagocytic cell, able to efficiently ingest lactobacilli, vaginal epithelial cells, leukocytes, and erythrocytes. Phagocytosis of leukocytes is another strategy for parasite to evade the cellular immune response. Phagocytosis of lactobacilli, which are responsible for maintaining the acidic pH of the vagina, would also enable the parasite to survive in more basic milieu (Rendón-Maldonado et al., 1998).

The ability of *T. vaginalis* to evade the host immune system is clearly an important aspect of its pathogenesis.

1.9. The Importance of Iron in Pathogenicity of *T. vaginalis*

Iron, which is an essential nutrient for the growth of many pathogens, is particularly important for *T. vaginalis*. Several reports have shown that iron can regulate a variety of trichomonal properties such as growth, and certain virulence traits (Lehker et al., 1991; Wilson and Britigan, 1998; Kim et al., 2006).

Using a mouse model, Ryu et al. (2001) showed that trichomonads cultured in regular Diamond's trypticase-yeast extract-maltose (TYM) and iron-supplemented TYM media could cause subcutaneous abscess in mice, while trichomonads grown in an iron-depleted medium failed to produce any pathology. This suggests that virulence of *T. vaginalis* is reduced under low iron conditions. Another report also demonstrated that trichomonal virulence increased following iron injection in mice (Budilova and Kulda, 1977).

The importance of iron for *T. vaginalis* was further demonstrated by the correlation of iron levels to the activity of *T. vaginalis* critical enzymes; high concentrations of iron in the medium are required to maintain maximal levels of hydrogenosomal enzyme activities (Gorrell, 1985). It has been suggested that iron could regulate the virulence of *T. vaginalis* by regulating hydrogenosomal activity through hydrogenosomal enzyme expression, and hydrogenosomal membrane potential (Kim et al., 2006). In addition, iron has a pivotal role in the expression of phosphohydrolases in *T. vaginalis*. When trichomonads are cultured in iron-depleted medium, both ecto-phosphatase and ecto-ATPase activities, which are considered as virulence factors, exhibit a remarkable reduction comparing to the control group (Jesus et al., 2006).

Iron levels influence a variety of properties of *T. vaginalis* including growth and multiplication, as well as protein-immunogen synthesis (Lehker and Alderete, 1992). It has been demonstrated that deferoxamine (DFO), a clinically approved iron chelator, has a potent and persistent inhibitory effect on viability and multiplication of *T. vaginalis*, in a drug concentration and time exposure-dependent manner. Furthermore, the proteinase activity of *T. vaginalis* grown for 48 hours in DFO inoculated TYM medium, showed a significant decrease at all drug concentrations applied in the study (Mahmoud, 2002).

T. vaginalis has the property of phenotypic variation as a mechanism of immune evasion. Alderete et al. (1992) reported that two classes of markers are alternately expressed on the surface of trichomonads: the highly immunogenic glycoproteins (P270) and the adhesins (AP65, AP51, AP33, and AP23). While all isolates (type I and II) synthesize and express P270 in the cytoplasm, only type II organisms can undergo phenotypic variation and express P270 on their cell surface. There is a relationship between the presence of the dsRNA virus and the ability of trichomonads to express immunogens on their surfaces and to

undergo phenotypic variation (Wang et al., 1987). Iron directly modulates the surface expression of P270 among virus-harboring organisms. Growth of virus-positive trichomonads in high-iron medium yields parasites without surface P270 (Alderete, 1999). On the other hand, iron regulates the synthesis and surface expression of four trichomonad adhesion proteins; increased amounts of adhesins were observed in trichomonads grown in high-iron medium (Lehker et al., 1991). These findings show that iron regulates the expression of at least two groups of protein markers on the surface of *T. vaginalis* (Garcia et al., 2003).

T. vaginalis resistance to complement-mediated lysis is also induced by incubation in high concentration of iron, most likely due to proteolytic cleavage of the complement component C3. It is an important strategy for *T. vaginalis* survival, since maximal resistance to complement may only be needed during menstruation, when trichomonads encounter high levels of complement and iron (Alderete et al., 1995b).

The above studies indicate that iron is a key element for regulating cellular activities in *T. vaginalis*.

1.10. Iron acquisition by *T. vaginalis*

Like almost all pathogenic organisms, iron is an essential nutrient for *T. vaginalis* (Ong et al., 2004). However, it is not readily available in vivo (Lehker and Alderete, 1992). As a host defence strategy against pathogens, the concentration of free iron in the extracellular environment is less than the amount required for pathogenic growth (Chen et al., 1996; Fouz et al., 1996). In order to survive, pathogenic microorganisms have developed sophisticated mechanisms to acquire iron from their host supplies; these mechanisms are also considered to be virulence factors (Wilson and Britigan, 1998; León-Sicarios et al., 2005). In

humans, iron is bound to the iron-binding glycoproteins lactoferrin (LF) and transferrin (TF), present in mucosal secretions and serum, respectively; or sequestered intracellularly as heme-containing proteins, such as hemoglobin and cytochrome (Lee, 1992). The acquisition of these host proteins by *T. vaginalis* through specific receptor-mediated mechanisms might represent strategies by which trichomonads obtain iron, and enhance their survival within the host environment (Peterson and Alderete, 1984).

Peterson and Alderete (1984) showed that *T. vaginalis* possess specific receptors for human lactoferrin, which is found in vaginal secretions. They showed that maximal lactoferrin binding is at 37°C, and that lactoferrin binding by *T. vaginalis* receptors results in intracellular iron accumulation and increased pyruvate/ferredoxin oxidoreductase activity. They suggested that the ability of trichomonads to bind human lactoferrin is important to the virulence of this parasite. Later Lehker and Alderte (1992) identified a 136 kDa lactoferrin-binding protein on the surface of *T. vaginalis* during its growth in iron-depleted medium. They reported that the trichomonad lactoferrin receptor is also regulated by iron. They showed that when trichomonads are grown in iron-depleted medium, the level of lactoferrin-binding activity increases. While the lactoferrin receptor is a minor component on the cell surface of trichomonads grown in normal medium, this receptor may be the major component of the cell surface of *T. vaginalis* depleted of intracellular iron pools. The ability of *T. vaginalis* to modulate the number and affinity of lactoferrin receptors in response to changing external and internal iron concentrations allows this parasite to regulate iron uptake at multiple levels (Lehker and Alderte, 1992). Lactoferrin influences resistance to complement-mediated lysis (Alderete et al., 1995b), and regulates the synthesis of four trichomonad adhesin proteins (Lehker et al., 1991). Lactoferrin is a key iron source for *T. vaginalis* (Alderete et al., 1995b). However, its concentrations in vaginal

fluids undergo dramatic changes during the menstrual cycle. The amount of lactoferrin is elevated after the postmenstrual phase and steadily decreased until menstruation (Lehker et al., 1991).

Transferrin, another iron-binding protein found in plasma, is not a source of iron for *T. vaginalis* (Lehker et al., 1991). It does not bind to *T. vaginalis* with high affinity and fails to compete with ^{125}I -labeled lactoferrin for binding to intact trichomonads (Peterson and Alderete, 1984). *T. vaginalis* is a non-invasive mucosal parasite (Gillin and Sher, 1981), and the ability of this parasite to bind to lactoferrin more effectively than plasma-derived transferrin confirms this finding.

Erythrocytes are another source of iron especially during menstruation when the level of lactoferrin is at its lowest. Trichomonads are able to completely phagocyte erythrocytes, and degradation of erythrocytes inside the phagocytic vacuoles has been reported (Rendón-Maldonado et al., 1998). As previously discussed, *T. vaginalis* also has a β -hemolytic activity (Krieger et al., 1983). Hemolysis results in the release of hemoglobin, which is the most abundant heme-containing protein in the body. It is a stable protein, formed by two α and two β chains, each one binding to a porphyrinic heme group, with iron in a ferrous state (Serrano-Luna et al., 1998). Free hemoglobin is readily bound by the serum protein haptoglobin and is not available in vivo (Lehker et al., 1990). Using ^{125}I -labeled hemoglobin, Lehker et al. (1990) showed that hemoglobin binds to *T. vaginalis* in a concentration-dependent manner. Binding of hemoglobin by the trichomonad receptors immediately after hemolysis, prevents released hemoglobin from being sequestered by host macromolecules. They suggested that the parasites bind to hemoglobin in a highly specific receptor-mediated fashion, and only unlabeled hemoglobin compete with the iodinated ligand, and both lactoferrin and transferrin were ineffective in a competitive binding assay. When hemoglobin

as a sole source of iron is added to the growth medium under iron-limiting conditions, *T. vaginalis* showed excellent growth comparing to the iron-chelated control medium (Min et al., 1998). This finding indicates that *T. vaginalis* is able to take up iron from hemoglobin and utilize this compound for its growth (Wilson and Britigan, 1998). Identification of a 60 kDa cysteine proteinase of *T. vaginalis*, which is capable of degrading hemoglobin into heme and globin, also implies that this parasite may use hemoglobin as a nutrient source of iron (Min et al., 1998).

It has been suggested that epithelial cells may be another source of iron for *T. vaginalis*. The squamous epithelial wall of the human vaginal cavity has an iron content, which varies throughout the hormonal cycle of the host (López et al., 2000). That erythrocytes and epithelial cell extracts stimulate growth in iron-limited medium strongly suggests that intracellular iron sources, such as cytochrome, ferritin, and hemoglobin, are important iron sources for *T. vaginalis* (Lehker and Alderete, 1992).

These different iron-acquisition systems, which are active depending on available iron sources, may be beneficial to *T. vaginalis* by providing alternative mechanisms for iron sequestration, and will allow for rapid adaptation to the changing host environment during establishment and maintenance of the infection (Lehker and Alderete, 1992; Alderete et al., 2004).

1.11. Experimental Rationale

The mechanisms of pathogenesis in *T. vaginalis* are multifactorial and virulence is greatly influenced by iron as it regulates growth rate, metabolic activities, and the expression of certain virulence phenotypes such as cytoadherence and resistance to complement lysis (Tsai et al., 2002; Garcia et al., 2003). Since there is no free iron in the vagina, it is not

surprising that *T. vaginalis* has evolved multiple mechanisms for acquiring iron through specific iron-binding (lactoferrin and ferritin) and iron-containing (hemoglobin and cytochrome) proteins (Lehker and Alderete, 1992). It has been demonstrated that *T. vaginalis* binds to lactoferrin by a specific 136- kDa receptor, which increases in number and affinity under iron-depleted conditions (Tachezy et al., 1996). Iron can also be obtained from hemoglobin following hemolysis. It has been suggested that multiple receptors with different affinities or a receptor with multiple binding sites with different affinities for hemoglobin might exist (Lehker et al., 1990). However, no specific hemoglobin-binding protein has been reported for *T. vaginalis* so far.

1.12. Hypothesis

Having different *T. vaginalis* isolates with different pathogenicity (as defined by human and mouse clinical presentation) in our lab, our first hypothesis was that the hemolytic activity of these isolates correlates with the virulence.

Our second hypothesis was that through specific hemoglobin receptor(s) on the surface of *T. vaginalis*, this parasite can utilize hemoglobin as a source of iron.

1.13. Objectives

The objectives of this project were as follows:

- (a) To study the hemolytic activities of four different clinical isolates of *T. vaginalis* by measuring the amount of hemoglobin released.
- (b) To identify a specific hemoglobin binding protein on the surface of *T. vaginalis* that enables the parasite to bind hemoglobin and uptake iron from this source.

CHAPTER TWO: MATERIALS AND METHODS

2.1. *T. vaginalis* strains

Four clinical isolates of *T. vaginalis* with different pathogenicity: OC15 (severe), 256 (moderate), DG (moderate), and 396 (asymptomatic), based on a standard scoring scheme evaluating clinical presentation (Garber and Lemchuk-Favel, 1990), were used in hemolysis study.

The moderate strain, DG, was used in one set of experiments, and the pathogenic isolate, OC15, was used for all experiments in identifying hemoglobin-binding protein(s). Isolates T016, antisense (AS) transfected T016, and sense (S) transfected T016 were kindly provided by Dr. John F. Alderete of University of Texas health science centre, San Antonio. Using the antisense technology, the expression of adhesin AP65 gene was reduced in AS transfected T016 isolate (Mundodi et al, 2004).

2.2. Growth media

Organisms were grown axenically from frozen stock in 15-ml glass screw-cap tubes containing 10 ml of Diamond's trypticase-yeast extract-iron (TYI-S-33) medium (pH 6.5) supplemented with vitamins (NCTC-109; Gibco/Invitrogen, Burlington, Ontario) - Tween 80 mixture, and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco/Invitrogen). AS- and S-transfected T016 isolates were cultivated in TYI medium in the presence of 75 µg/ml or 100 µg/ml Neomycin (Sigma Chemical Co., Oakville, Ontario) from a sterilized stock solution of 10 mg/ml.

Low-iron medium was prepared by adding iron chelator, 2,2-dipyridyl (2,2-DP) (Sigma), at 100 µM final concentration to the TYI growth medium (Lehker et al., 1991; Ryu et al., 2001). A 2 M stock solution of 2,2-DP was made in dimethylsulfoxide (Sigma), and

filter sterilized. Low-iron parasites were obtained by growing trichomonads in low-iron medium supplemented with 2.5% (v/v) of FBS for at least four days with subculturing every 48 hours. For parasites grown in high-iron concentrations, ammonium ferric citrate (BDH chemicals/VWR, Mississauga, Ontario) at 250 μ M final concentration was added to the TYI medium, supplemented with 10% of FBS (Lehker et al., 1991; Alderete et al., 1995b). To make Hemoglobin added TYI medium, human hemoglobin (Sigma) at 5 μ M final concentration (from a 100 μ M stock solution made in sterile double distilled water), was added to the low-iron medium, and the medium was filter sterilized. Iron-deficient trichomonads were grown in hemoglobin added medium supplemented with 2.5% FBS.

Cultures were incubated at 37°C in a 5% CO₂ atmosphere (Forma Series II Water Jacketed CO₂ incubator; Fisher Scientific, Ottawa, Ontario), and passaged every two to three days. Frozen axenic stocks were prepared by harvesting trichomonads in logarithmic growth phase, and resuspending them in fresh TYI with 10% FBS and 10% (v/v) dimethylsulfoxide (DMSO). The preparations were then mixed, aliquoted into 1.5 ml cryovials, and frozen at -80°C or in liquid nitrogen.

2.3. Hemolysis Assay

In this experiment, sheep blood in Alsevers solution (Oxoid, Nepean, Ontario) was used. The plasma was carefully removed by centrifugation (Jouan CR3i-V1) at 250 x g for 10 minutes at 4°C, and the erythrocytes were washed three times in sterile phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.2). Trichomonads in their log phase were harvested and washed three times with cold PBS by centrifugation (750 x g, 10 minutes at 4°C). Viable trichomonads were counted with trypan blue on a hemocytometer slide (Hausser Scientific, Horsham, PA, USA)

under an inverted microscope (Olympus CK2). A volume of 50 μ l of washed undiluted erythrocytes was mixed with 2.5 ml of filter sterilized Hank's balanced salt solution (HBSS) (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, and 4.2 mM NaHCO₃) with glucose (1.0 g/L) pH 5.8 containing a total of 1×10^6 trichomonads. In addition, 50 μ l of washed erythrocytes was added to 2.5 ml sterile double distilled water as a positive control, and another 50 μ l of washed erythrocytes was added to 2.5 ml HBSS as a negative control. Control tubes were included in all assays. All tubes were incubated at 37°C in 5% CO₂ for 18 hours. After the incubation period, the mixture was centrifuged ($250 \times g$ for 10 minutes at 4°C).

The absorbance of the supernatants (samples and controls) were measured at 540 nm with a spectrophotometer (model Genesys 20, Thermo Spectronic). The results were expressed as percentages of total hemolysis (100%). The percentage of hemolysis in each tube was determined by using the following equation: % He = (Ex HR – SHR)/(MHR - SHR) x 100, where % He is the hemolysis percentage, ExHR the experimental hemoglobin release, SHR the spontaneous hemoglobin release (negative control), and MHR the maximum hemoglobin release (positive control) (Vargas-Villarreal et al., 2003). The mean and the standard error of the hemolytic activity of each *T. vaginalis* isolate was calculated after performing the assay six times, each in triplicate.

The association between *T. vaginalis* hemolytic activity and virulence was determined by Student's *t* test with significance level $P < 0.05$.

2.4. Membrane Extraction

The first step in studying hemoglobin-binding protein(s) on the surface of trichomonads was extracting the membrane, which was done by following two different

protocols. For all experiments, only trichomonads in mid- to late logarithmic growth phase were used. Parasites were washed three times in cold PBS (pH 7.2) to remove loosely bound medium components, and were counted on a hemocytometer slide. Their viabilities were evaluated applying the trypan blue dye exclusion test.

The first protocol was described by Sengupta et al. (1999) for preparing membrane fractions from *Leishmania*. Washed trichomonads were resuspended in hypotonic Tris-HCl buffer (5mM, pH 7.2) to a density of 2×10^8 cells/ml. After incubation for one hour at 4°C, the cells were broken by sonication (3 x 5 s) with an output of 15 watts on ice using a sonicator (Model 60 Sonic Dismembrator, Fisher Scientific). The unbroken cells and nucleus were separated by low speed centrifugation (200 x g for 10 minutes at 4°C). The supernatant was then transferred to 1.5 ml snap-cap ultracentrifuge tubes (Beckman, Fullerton, CA, USA), and centrifuged at 100,000 x g (TL-100 ultracentrifuge, Beckman) for one hour at 4°C. The supernatant was discarded and the pellet in each tube was resuspended in 300 µl PBS (pH 7.2) containing 2% β-octyl glucoside (Sigma) and kept at 4°C overnight. The suspension was centrifuged at 100,000 x g for one hour at 4°C to separate membrane proteins from debris. The supernatant, which was transferred into new Eppendorf tubes, contained extracted membrane proteins.

The second protocol used was slightly modified from that described by Moreno-Brito et al. (2005) for preparation of *T. vaginalis* cell fractions. Washed trichomonads (2×10^8 cell/ml) were suspended in ice-cold buffer R (20 mM Tris, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 1 mM EDTA and 1 mM β-mercaptoethanol). Proteinase inhibitors TLCK and leupeptin (both from Sigma) were prepared as stock solutions in double distilled water, and 1 mM TLCK and 0.2 mM leupeptin (final concentration) were added to the cell suspension to inhibit cysteine proteinases released by live trichomonads. Parasites were manually

homogenized in a glass Dounce homogenizer (pestle B) (Fisher Scientific) on ice with 20-25 strokes, and nuclei and cellular debris were separated by centrifugation at 2000 x g for 10 minutes at 4°C. The total-protein extract was then transferred to ultracentrifuge microtubes and centrifuged for 1.5 hour at 25,000 x g at 4°C. Membrane proteins were recovered by resuspending the pellet in buffer R (without β -mercaptoethanol).

The protein concentration was estimated by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) (Pierce) as a standard. The proteins were stored at -20°C until further use.

In some experiments *T. vaginalis* total proteins was needed. Therefore, a trichloroacetic acid (TCA) precipitation method, as described by Peterson and Alderete (1982), was used. Approximately 10^7 trichomonads/ml were washed in cold PBS (pH 7.2), and suspended in 2.5 ml cold PBS with 10% (w/v) trichloroacetic acid (Sigma). The suspension was incubated at 4°C for at least four hours. After the incubation period the suspension was centrifuged at 11,000 x g for five minutes at 4°C, and the supernatant was discarded. The acid precipitated material was washed with cold PBS (pH 7.2) three times to remove excess TCA, and was finally dissolved in 2 x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer consisting of 0.125 M Tris-hydrochloride (pH 6.8), 5% (v/v) β -mercaptoethanol, 20% (v/v) glycerol, 4% (w/v) SDS, and 0.01% (w/v) bromophenol blue as the tracking dye. Samples were boiled for three minutes, and undissolved residues were removed by centrifugation (11,000 x g for five minutes). The supernatant was aliquoted into fresh microfuge tubes and kept at -20°C until further use.

2.5. Concentrating/Desalting Proteins

In some experiments the concentration of membrane proteins was low. To have more concentrated proteins, two different filters were used. Microcon centrifugal filter devices (YM - 3) from Millipore (Mississauga, Ontario, Canada) were used to concentrate small (10-500 μ l) volumes of proteins according to the manufacturer's protocol. Each filter device comprised of one sample reservoir, which was inserted into a vial. The protein was pipetted into the sample reservoir (maximum 500 μ l), counterbalanced with a similar device, and centrifuged at 10,000 $\times g$ for one hour at 4°C. The sample reservoir, which contained concentrated protein, was placed upside down in a new vial (1.5 ml Eppendorf tube), and centrifuged at 1000 $\times g$ for three minutes at 4°C to transfer concentrated protein to vial.

For higher volumes (up to 4 ml), Amicon ultra-4 centrifugal filter devices (Millipore) were used, which contained of a cap, filter unit, and a 15 ml centrifuge tube. First the filter was washed with 3 ml PBS (pH 7.2) for 10 minutes at 2000 $\times g$ at room temperature. PBS was discarded and the protein sample was added to the filter unit. Depending upon the concentration needed, protein samples were centrifuged at 2000-4000 $\times g$ for 10 minutes at 4°C. The concentrated protein was removed by inserting a micropipette into the bottom of the filter unit and withdrawing the sample using a side-to-side sweeping motion to ensure total recovery.

In biotinylation of hemoglobin experiments, these filters were used as a desalting device to remove excess biotin (will be discussed later).

2.6. Biotinylation of trichomonad membrane proteins

Cell surface biotinylation is a powerful tool that is used to selectively label proteins found on the plasma membrane. Biotin is a small naturally occurring vitamin that binds

with high affinity to avidin and streptavidin proteins. Because of its small size (244 Da), biotin can be covalently bound to many proteins without altering their biological activity. The labelled molecule can be detected by dot blot or Western blot using streptavidin or avidin probes (Daniels and Amara, 1998). N-hydroxysuccinimido-biotin (NHS-biotin), the popular biotinylation reagent, is not permeable to live cells and is incorporated onto surface proteins through their lysine residue (Min et al., 1994).

Cell surface proteins of intact trichomonads were biotinylated following the same procedure previously described for *Leishmania* (Sengupta et al., 1999). Trichomonads were washed three times with cold PBS (pH 7.2). Washed cells were then suspended in 1 ml PBS (pH 7.2) with 0.5 mg/ml NHS-biotin (Sigma) final concentration. NHS-biotin stock (200 mg/ml) was made by dissolving NHS-biotin in DMSO, aliquoted into microfuge tubes and stored at -20°C . After one-hour incubation at 4°C , trichomonads were washed three times with ice-cold PBS (pH 7.2) containing 50 mM NH_4Cl (Sigma) and once with ice-cold PBS (pH 7.2) to eliminate free biotin.

In later experiments No-Weigh™ NHS-PEO₄-Biotin (Pierce), in which NHS-PEO₄-Biotin was packaged in convenient pre-measured microcentrifuge tubes (8 x 2 mg), was used. Since this reagent was water soluble, reactions were performed in the absence of organic solvents like DMSO. Following manufacturer's protocol, trichomonads were washed three times with cold PBS (pH 8.0). Cells were suspended in PBS (pH 8.0) at a concentration of 2×10^7 cells/ml. NHS-PEO₄-Biotin (Pierce) were stored at 4°C . Immediately before use, 200 μl double distilled water were added to 2 mg of NHS-PEO₄-Biotin and mixed by pipetting up and down. Fifty μl of the solution were added to one ml of trichomonad suspension, and the reaction was incubated for 30 minutes at room temperature.

Trichomonads were then washed once with 50 mM Tris (pH 8.0), and three times with ice-cold PBS (pH 8.0) to quench and remove excess biotin reagent.

In both protocols, trichomonads viability was assessed by the trypan blue exclusion method before and after biotin treatment. Membrane fractions of these biotinylated trichomonads were prepared as described previously.

2.7. Biotinylation of hemoglobin

Biotinylated human hemoglobin was used as a probe to detect hemoglobin-binding protein(s). Human hemoglobin (Sigma) was biotinylated using EZ-Link[®] Sulfo-NHS-LC-Biotinylation kit (Pierce). Hemoglobin was dissolved in double distilled water (4 mg/ml final concentration), mixed at room temperature for one hour, and centrifuged at 750 x g for 10 minutes at room temperature to pellet undissolved hemoglobin. The supernatant was then filtered through a 0.45- μ m-pore-size sterile 25mm-syringe microfilter (Fisher Scientific).

The amount of biotin reagent used in each reaction was calculated according to the manufacturer's protocol. A 10 mM fresh stock of Sulfo-NHS-LC-Biotin was made immediately before use, by adding 2.2 mg Sulfo-NHS-LC-Biotin to 400 μ l of double distilled water. For 4 mg/ml Hemoglobin (molecular mass 64.5 kDa) with a final volume of 2 ml, 250 μ l of Sulfo-NHS-LC-Biotin solution was added. The reaction was incubated either on ice for two hours or at room temperature for 30 minutes. In later experiments No-Weigh[™] NHS-PEO₄-Biotin (Pierce) was used. The protocol was the same as described above, except for the amount of biotin reagent used, since the two reagents had different molecular weights. Therefore, for biotinylation of 2 ml hemoglobin solution (4 mg/ml final concentration), 145 μ l of 10 mg/ml NHS-PEO₄-Biotin solution was added.

Zeba™ desalt spin column, 5 ml (Pierce), was used for removing the excess biotin. The column was placed in a 15 ml conical collection tube and centrifuged at 1,000 x g for five minutes at room temperature to remove the storage solution. The column was placed in a new collection tube and the biotinylated hemoglobin was slowly applied to the center of the compact resin bed. As a stacker, 2.5 ml of double distilled water was also added to the resin after the biotinylated hemoglobin had fully absorbed. The column was centrifuged at 1,000 x g for five minutes at room temperature. The desalted sample was collected in a 15 ml conical collection tube. To concentrate biotinylated hemoglobin, and to remove all traces of non-reacted biotinylation reagent, Amicon ultra-4, centrifugal filter devices (Millipore) were used (as described previously).

To estimate the extent of biotinylation and ratio of biotin to protein, avidin-HABA assay was performed. It has been shown that the absorption of avidin-HABA complex at 500 nm (A_{500}) decreases proportionally with increased concentration of biotin as the HABA dye (4'-hydroxyazobenzene-2-carboxylic acid) is displaced from avidin due to the higher affinity of avidin for biotin. Following the protocol provided in the EZ-Link® Sulfo-NHS-LC-Biotinylation kit (Pierce), HABA-avidin solution was made by adding 2.5 mg avidin, and 150 μ l HABA to 4.8 ml PBS (pH 7.2). PBS was used as a blank to zero the spectrophotometer (model Genesys 20, Thermo Spectronic). The absorbance of 900 μ l HABA-avidin solution was measured at 500 nm. 100 μ l of biotinylated hemoglobin was added to the cuvette containing HABA-avidin and mixed well. The absorbance of the solution was measured at 500 nm, and the value was recorded as A_{500} HABA/Avidin/Biotin sample. The number of moles of biotin per mole of hemoglobin was calculated according to the protocol supplied by the manufacturer (Pierce, product number 21435).

2.8. Affinity chromatography of the membrane proteins

Hemoglobin-binding proteins were isolated by affinity chromatography method using hemoglobin-agarose beads purchased from Sigma (100 μ l of packed gel containing 1.2 mg hemoglobin). Hemoglobin-affinity chromatography was performed as described previously (Fouz et al., 1997; Sengupta et al., 1999) with the following modifications. Fifty μ l of hemoglobin-agarose beads (stored at 4°C) were washed with a low-salt buffer (25 mM Tris-HCl, 100mM NaCl, pH 7.4). Washes were performed three times by resuspending the hemoglobin-agarose beads in 400 μ l buffer and centrifugation at 4000 $\times$ g for five minutes at 4°C. One hundred μ l of membrane protein (the concentration was ranged from 0.25 to 1 mg/ml), either biotinylated or non-biotinylated, was added to the washed hemoglobin-agarose beads in a 1.5 ml microcentrifuge tube. As a control, 100 μ l buffer, the same buffer used for diluting membrane proteins (PBS or buffer R), was added to 50 μ l washed beads. Binding reaction mixtures were incubated on a rotator for three hours at room temperature. The beads were pelleted by centrifugation at 4000 $\times$ g for five minutes at room temperature, and subsequently washed twice (10 minutes each wash) with three different buffers (50 mM Tris-HCl, 100mM NaCl, pH 7.4; 50 mM Tris-HCl, 1M NaCl, pH 7.4; and 50mM Tris-HCl, 1 M NaCl, 0.25 mM EDTA, pH 7.4) to remove proteins that bound non-specifically to the beads. The last wash (sixth wash) fraction in each tube was concentrated by using the Microcon centrifugal filter devices (Millipore), as described above. Fifty μ l of 2 $\times$ SDS-PAGE sample buffer was added to the washed beads and boiled at 100 °C for five minutes. In experiments with biotinylated membrane proteins, β -mercaptoethanol was not added to the sample buffer, since it could reduce the disulfide bonds and release biotin from proteins. The samples were allowed to cool to room temperature, and then the beads were harvested by centrifugation at 10,000 $\times$ g for five minutes at room temperature. The supernatants,

which contained the eluted proteins, were transferred to clean microcentrifuge tubes, and were analyzed later by SDS-PAGE, and Western blot (in some experiments).

Hemoglobin-binding proteins could also be isolated by affinity chromatography purification using immobilized streptavidin (Pierce) and biotinylated hemoglobin. The first step was to incubate 50 μ l membrane proteins with 50 μ l biotinylated human hemoglobin (5 μ M) on a rotator for two hours at room temperature. Subsequently, 100 μ l of streptavidin beads, which were equilibrated to room temperature, were washed three times with PBS (pH 7.2) by centrifuging at 4000 $\times$ g for five minutes at room temperature. Membrane proteins and biotinylated hemoglobin were then added to the washed streptavidin beads in a 1.5 ml microcentrifuge tube and incubated on a rotator for one hour at room temperature. As a control, 100 μ l of PBS (pH 7.2) was added to the washed streptavidin beads and incubated in parallel with the experimental samples. The streptavidin-bound complex were washed six times with PBS (pH 7.2), and proteins were eluted by adding 50 μ l 2 $\times$ SDS-PAGE sample buffer to the washed beads and boiling them for five minutes. The rest was the same as described for hemoglobin-agarose beads.

To identify any heme-binding protein in *T. vaginalis* membrane protein, affinity chromatography using hemin-agarose (Sigma) was performed as described by Dashper et al. (2000) with some slight changes. Briefly, 20 μ l of hemin-agarose beads was washed three times with a low-salt buffer (100 mM NaCl, 25 mM Tris-HCl, pH 7.4), and 100 μ l of membrane protein was added to the washed agarose beads. As a control, 100 μ l of the buffer was also added to the washed hemin-agarose beads. The mixture was incubated on a rotator at room temperature for three hours. The hemin-agarose was washed six times with the same buffer described above, and bound proteins were eluted by adding 50 μ l 2 $\times$ SDS-

PAGE sample buffer to the pellet, and boiling for five minutes. Eluted proteins were analyzed by SDS-PAGE.

2.9. Competition binding assay

The specificity of the binding activities of the heme/hemoglobin-binding proteins was examined in competition experiments using different concentrations of hemin or hemoglobin. The hemin solution was made by dissolving bovine hemin chloride (Sigma) in filter-sterilized 0.1 NaOH.

Membrane preparations were pre-incubated with different concentrations of hemoglobin or hemin (ranging from 10 μ M to 1mM) on a rotator for one hour at room temperature before being applied to hemin- or hemoglobin-agarose affinity chromatography.

2.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Eluted proteins, concentrated last washes from affinity chromatography, and membrane proteins were analysed by SDS-PAGE. Membrane proteins (0.2 mg/ml) and concentrated last washes were mixed with 2 x SDS-PAGE sample buffer, and boiled for 5 minutes. A ready load protein ladder (BenchMark™ Protein Ladder from Invitrogen, Burlington, Ontario) was used as a molecular weight standard for SDS-PAGE.

The separating gel consisted of 1.5 M Tris (pH 8.8), 10% (w/v) Ammonium persulfate, 10% (w/v) SDS, TEMED, H₂O, and 7.5% to 10% (w/v) acrylamide (Protogel; 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide; National Diagnostics, Atlanta, GA, USA). Stacking gel consisted of 1 M Tris (pH 6.8), 10% (w/v) Ammonium persulfate, 10% (w/v) SDS, TEMED, H₂O, and 3% (w/v) acrylamide (Protogel). Samples (20 to 50 μ l) were loaded onto the gel and electrophoresis was carried out at 120 V in Tris-glycine SDS-

PAGE running buffer (25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS) using the Bio-Rad (Mississauga, ON) Mini-Protein II Electrophoresis cell system. After electrophoresis, proteins were stained by soaking the gels in Coomassie Brilliant Blue stain containing 40% (v/v) methanol (Fisher Scientific), 7% (v/v) glacial acetic acid (Fisher Scientific), 0.1% (w/v) Coomassie brilliant blue R-250 (EM Science, Darmstadt, Germany), and 0.02% (w/v) Bismarck brown R (Sigma), and gently shaken for one hour at room temperature. Gels were destained overnight in destaining solution (40% (v/v) methanol, and 7% (v/v) glacial acetic acid), and were then washed with distilled water.

In later experiments, gels were stained using RAPIDstain (MJS BioLynx Inc., Brockville, Ontario), according to the manufacturer's protocol. After electrophoresis, gels were washed three times in double distilled water and stained with RAPIDstain for one hour, and destained with double distilled water overnight. All steps were performed at room temperature. Gels were preserved by air-drying between two moistened cellophane sheets (Research Products International Corp., Mount Prospect, IL, USA) in gel drying frames.

In some experiments, gels were stained using SYPRO Ruby protein gel stain (Sigma), which offers sub-nanogram sensitivity. After fixing gels in fixer (50% (v/v) ethanol and 2.5% (v/v) glacial acetic acid), they were placed in SYPRO Ruby stain, covered with aluminum foil to protect from light, and gently shaken overnight. Gels were washed in double distilled water, and proteins were visualized under UV illumination.

Protein samples were also analysed by nondenaturing SDS-PAGE (NDS-PAGE), in which β -mercaptoethanol and SDS were omitted from the sample buffer. SDS was also not included in running buffer, stacking, and separating gels, and protein samples were not denatured by boiling prior electrophoresis.

2.11. Western blot and Dot blot analysis

To detect the biotinylated surface proteins of *T. vaginalis*, proteins were transferred from SDS-PAGE gel onto polyvinylidene fluoride (PVDF) membrane (Immobilon-FL transfer membrane, 0.45 μm pore-size, Millipore) using a Trans-Blot semi-dry electrophoretic transfer apparatus (Bio-Rad). Prior to electroblotting, gel, membrane, and filter papers were equilibrated in fresh Towbin transfer buffer (25 mM Tris base, 192 mM Glycine, and 20% (v/v) methanol) for 30 minutes. The apparatus was assembled according to the instructions provided by manufacturer, and the transfer was performed at 20 V for 45 minutes at room temperature. The transfer was confirmed with Ponceau S staining (0.2% (w/v) Ponceau S (Sigma) and 1% (v/v) glacial acetic acid) of the membrane.

The membrane was blocked with 5% (w/v) skim milk in PBS (pH 7.2) and 0.1% (v/v) Tween 20 (Sigma) (PBS-T) with gentle shaking for one hour at room temperature. The paper was washed three times each for 10 min with PBS-T, and were incubated in avidin-horseradish peroxidase (avidin-HRP) (Sigma) at a dilution of 1:5000 in PBS-T at room temperature overnight. The avidin-HRP stock (1 mg/ml) was made by dissolving it in 10 mM PBS (1.9 mM NaH_2PO_4 , 8.1 mM Na_2HPO_4 , and 150 mM NaCl; pH 7.2), and was stored at -20°C . The paper was washed with three changes of PBS-T (10 minutes each), and was developed with a solution consisting of α -chloronaphthol (Sigma) in methanol (0.3 mg/ml), PBS, and 30% (v/v) hydrogen peroxide.

Another way to detect hemoglobin-binding protein(s) was to use biotinylated hemoglobin as a probe in Western blot assay. As discussed above, the separated proteins (membrane and total *T. vaginalis* proteins) were transferred from gel to a membrane, and blocked as above. Then the membrane was incubated in blocking buffer (2% skim milk (w/v) in PBS-T) containing biotinylated human hemoglobin (50 nM) for two to three hours

at room temperature with gentle shaking. The membrane was then washed three times before incubating with avidin-HRP at a 1: 5000 dilution in PBS-T overnight. Following three washes with PBS-T, the proteins were detected using α -chloronaphthol/ hydrogen peroxide mixture. The membrane was washed with distilled water to stop the reaction.

The protocol described by Guan et al. (2002) to identify hemoglobin-binding proteins was also used with slight modifications. *T. vaginalis* membrane extracts and total proteins were separated on 10% SDS-PAGE gel, transferred to PVDF membrane, and blocked. The membrane was incubated with human hemoglobin (15 μ M) in blocking buffer (2% skim milk in PBS-T) for three hours at room temperature. After washing three times with PBS-T, the membrane was incubated with 2% blocking buffer containing 1: 1000 rabbit anti-human hemoglobin antiserum (Sigma) overnight at 4°C with gentle shaking. After washing, the membrane was incubated with 1: 5000 or 1: 10,000 dilutions of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma) for one hour at room temperature, and developed with TMB membrane peroxidase substrate (KPL, Gaithersburg, MD, USA). The reaction was stopped after one to three minutes by rinsing the membranes in distilled water for 10-20 seconds.

Hemoglobin binding was also assayed in *T. vaginalis* membrane extracts using a dot blot method. Five μ l membrane proteins were manually blotted onto Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was then air-dried, blocked, and tested for hemoglobin-binding with biotinylated hemoglobin or anti-hemoglobin antibody, as described above.

2.12. Peptide sequencing

Stained heme- and hemoglobin-binding protein bands affinity-purified by either heme- or hemoglobin-agarose were excised from the gels. In order to protect gels from Keratin contamination by human hands and from dust in the air, gloves were worn all the time, and proteins were excised in a laminar flow hood. Clean glassware and reagents were also used to minimize contamination. The excised band was sliced into roughly 1 mm pieces and transferred to a clean, sterile 1.5 ml microcentrifuge tube that had been rinsed with ethanol (70%) and dried before use. A minimal amount of 1% (v/v) acetic acid was added to cover the gel cubes. The microcentrifuge tube was stored at 4°C until it was submitted to Ontario Genomics Innovation Centre (Ottawa Health Research Institute, Ottawa, Ontario) for protein identification. The proteins were then subjected to tryptic digestion, and the peptides obtained were purified and sequenced by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Subsequently the mass spectrometry results were processed using Mascot (Matrix Science, Boston, MA, USA) database search system. Amino acid sequences were also analysed with the Basic Local Alignment Search Tool (BLAST) program at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>) for the homology search.

CHAPTER THREE: RESULTS

3.1. Hemolytic activity of *T. vaginalis* isolates

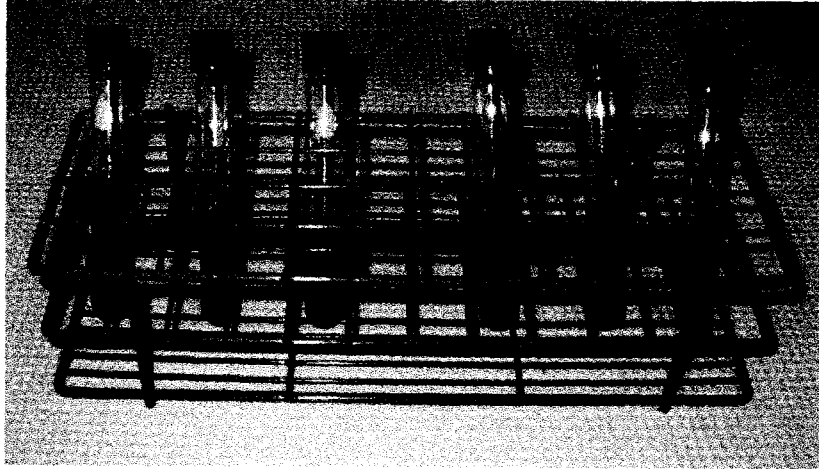
After 18-hour incubation of sheep red blood cells with trichomonads, results showed that all four *T. vaginalis* isolates regardless of clinical pathogenicity levels were capable of lysing red blood cells (Figure 1). The percentage of hemolysis of each isolate was determined as described in Materials and Methods. As can be seen in Table 1, there was no significant difference between levels of hemolysis in different *T. vaginalis* isolates with different clinical pathogenicity.

Results also showed that DG isolate, which had been able to induce experimental vaginitis in mice but has lost its pathogenicity due to prolonged cultivation, had similar hemolytic activity as a severe, fresh clinical isolate like OC15. Therefore, hemolytic ability was maintained in *T. vaginalis* even after long-term passaging in axenic culture, and did not appear to correlate with clinical pathogenicity.

Figure 1. Hemolytic activity in different clinical *T. vaginalis* isolates.

- A. Sheep red blood cells were added to four different trichomonad strains along with negative (sheep red blood cells in HBSS) and positive control (sheep red blood cells in double distilled water).
- B. After 18-hour incubation time at 37°C in a 5% CO₂ atmosphere, red blood cells were lysed in positive control and trichomonad samples. Spontaneous hemoglobin release was also observed in negative control.

A



B

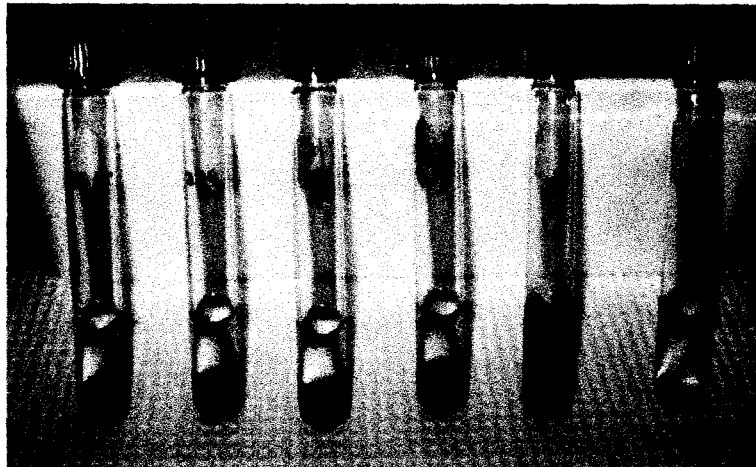


Table 1. The hemolytic activity of four isolates of *T. vaginalis* had no correlation with virulence.

- A. Mean $\pm$ SD of 6 triplicate samples (n=18). % hemolysis is compared to positive control.
- B. Means of paired isolates were compared using Student's t-test. In all cases the calculated t-value was less than the t-value in the Standard table of significance when $p=0.05$. Therefore, no significant difference ($p<0.05$) between hemolytic activity of different *T. vaginalis* isolates was observed.

A

	OC15	DG	256	396
Virulence	Severe	Moderate	Moderate	Asymptomatic
Hemolytic activity Mean (%) $\pm$ SD	33.1 $\pm$ 7.3	31.2 $\pm$ 6.3	30.7 $\pm$ 5.3	26.8 $\pm$ 12.02

B

	OC15 and DG	OC15 and 256	OC15 and 396	DG and 396	256 and 396
t-value	0.84	1.36	1.9	1.11	1.26
Degree of freedom (n_1+n_2-2)	34	34	34	34	34
Standard t-value ($P=0.05$), $n=30$	2.04	2.04	2.04	2.04	2.04
Significant difference ($P<0.05$)	—	—	—	—	—

3.2. *T. vaginalis* grown in different iron concentrations

Trichomonads grown at least 24 hours in low-iron medium, showed slower growth rate comparing to those grown in normal and high-iron conditions. The amount of heat-inactivated fetal bovine serum (FBS) added to their medium had an important impact in their growth rate. Addition of 10% FBS to the culture medium caused trichomonads to grow almost as well as control ones (trichomonads grown in normal iron concentration). However, lowering this amount to 2.5% caused a remarkable decrease in their growth rate. At FBS concentrations lower than 2.5%, *T. vaginalis* growth was almost completely arrested. Therefore, to grow trichomonads under iron-depleted condition, the minimum amount of FBS was added to their growth culture. The same results were obtained when trichomonads were grown in low-iron medium supplemented with human hemoglobin. When hemoglobin was added to iron-depleted trichomonads, after 24-hour incubation the released heme was detectable in growth medium, forming a visible brownish pellet. Similar reactions were not observed in controls (hemoglobin-supplemented low-iron medium, and trichomonads grown in normal growth medium). This result confirmed that trichomonads are able to degrade hemoglobin under iron depleted growth conditions (Min et al., 1998).

3.3. *T. vaginalis* membrane extraction

T. vaginalis like many other pathogenic organisms cannot synthesize heme. In the host, hemoglobin is the most important reservoir of heme. It is possible that *T. vaginalis* has evolved receptors for heme and/or hemoglobin on its surface. Therefore, the first step in identifying heme- and/or hemoglobin-binding proteins is to extract *T. vaginalis* membrane. In this study, two different membrane extraction protocols were applied. In the first protocol, trichomonads, like many other microbial pathogens, were lysed using a

sonicator. However, the result was not satisfying. It seemed that *T. vaginalis* has a fragile membrane and sonication was a harsh method for lysing trichomonads. Therefore, the sonication time was decreased to less than five seconds each pulse. Later, the membrane fraction was prepared by gentle homogenization, following the protocol used by Moreno-Brito et al. (2005), which was specific for *T. vaginalis*.

3.4. Hemoglobin-binding by *T. vaginalis* membrane extract

T. vaginalis membrane extract was assayed for hemoglobin-binding activity using a dot blot technique. As described in Materials and Methods, dot blot assay was carried out using two different protocols: with biotinylated human hemoglobin, and with anti-human hemoglobin antiserum. The results in both protocols showed that *T. vaginalis* membrane proteins were able to bind hemoglobin, and confirmed the previous suggestion of the presence of hemoglobin receptor(s) in *T. vaginalis* (Lehker et al., 1990; Alderte et al., 2004).

The membrane proteins bound to biotinylated hemoglobin with the intensity proportional to their concentrations blotted onto the nitrocellulose paper (Figure 2). To reassure that the binding is specific and no free biotin was present in biotinylated hemoglobin preparation, bovine serum albumin (BSA) was blotted onto the membrane as a control. The result was always negative. Non-specific binding of either the biotinylated hemoglobin or avidin-HRP conjugate to the nitrocellulose paper was also not detected, indicated that the observed hemoglobin binding was specific (Figure 2).

No difference in hemoglobin binding was observed between membrane extracts of trichomonads grown in normal, low-iron, high-iron, and hemoglobin added medium in dot blot assay using anti-human hemoglobin antiserum as a probe (Figure 3). A similar

Figure 2. Dot blot assay demonstrating binding of biotinylated human hemoglobin to membrane proteins of *T. vaginalis*. Membrane proteins bound to biotinylated hemoglobin in a concentration dependent manner.

A1: *T. vaginalis* membrane extract (0.75 mg/ml), A2: membrane extract (1.5 mg/ml), A3: membrane extract (3 mg/ml), A4: membrane extract (6 mg/ml).

B1: Bovine serum albumin (BSA) (2 mg/ml), B2: BSA (1 mg/ml), B3: PBS, B4: PBS (Negative controls).



B

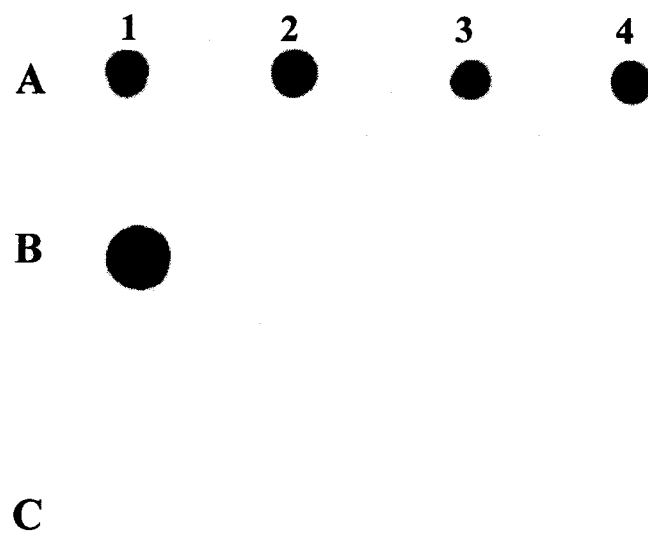
Figure 3. Dot blot assay showing that *T. vaginalis* was able to bind hemoglobin, regardless of the iron level in the growth medium. Membrane extracts (1 mg/ml) from trichomonads grown in different iron concentrations were blotted onto nitrocellulose membrane, and incubated with human hemoglobin solution (15 μ M). The bound hemoglobin was probed with rabbit anti-human hemoglobin antiserum followed by HRP-conjugated goat anti-rabbit IgG. Hemoglobin-binding was visualized by TMB membrane peroxidase substrate.

A1: membrane extracts from trichomonads grown in normal medium, A2: low-iron condition, A3: high-iron condition, and A4: hemoglobin-added TYI.

B1: Hemoglobin (1mM) (antiserum control), B2: PBS, B3: PBS (Negative controls)

C1: membrane extract from trichomonads grown in TYI without pre-incubation with human hemoglobin (control for non-specific binding).

No reaction was detected in B2, B3, and C1 controls.



experiment using biotinylated hemoglobin as a probe confirmed that all trichomonads, regardless of the iron level in the growth medium, were able to bind hemoglobin (data not shown).

3.5. Identification of hemoglobin-binding proteins

To identify *T. vaginalis* hemoglobin-binding protein(s), membrane preparations of trichomonads grown in normal TYI medium was purified by affinity chromatography using hemoglobin-agarose beads. Hemoglobin-binding proteins have been identified in some pathogenic bacteria (Archambault et al., 2003; Furano et al., 2005; Guan et al., 2004), and protozoan parasites like *L. donovani* (Sengupta et al., 1999) applying the same technique.

The eluted protein fractions were analysed by SDS-PAGE, revealing the presence of two bands of approximately 48 kDa, and another of approximately 63 kDa (Figure 4, lane 3). Eluted hemoglobin was observed in control (hemoglobin-agarose beads incubated with buffer) (Figure 4, lane 4). No bands were detected in the concentrated last wash sample (Figure 4, lane 5).

To confirm this finding, immobilized streptavidin and biotinylated hemoglobin were also used to detect hemoglobin-binding proteins. As described in Materials and Methods, *T. vaginalis* membrane extract was first incubated with biotinylated hemoglobin, and then was subjected to affinity purification with streptavidin-agarose beads. The eluted proteins were analysed by SDS-PAGE. The result was similar to that obtained before. Two bands of approximately 48 and 63 kDa were observed (Figure 5A, lane 3; Figure 5B, lane 2). No bands were detected in streptavidin control (Figure 5A, lane 4; Figure 5B, lane 3) and concentrated last washes (Figure 5A, lane 5; Figure 5B, lane 4).

Figure 4. Identification of hemoglobin-binding proteins of *T. vaginalis* strain OC15 using hemoglobin-affinity chromatography. Membrane extracts were incubated with hemoglobin-agarose beads; the hemoglobin-binding proteins were then eluted and subjected to SDS-PAGE (3% stacking, 10% separating gel). Gel was stained with Coomassie brilliant blue.

Molecular mass protein marker in kDa (lane 1); *T. vaginalis* membrane extract (lane 2); hemoglobin-agarose affinity-purified membrane proteins from *T. vaginalis* (lane 3); hemoglobin-agarose control (lane 4); concentrated last wash (lane 5).

Asterisks indicate the position of the 48- and 63-kDa hemoglobin-binding proteins. Arrowhead indicates the hemoglobin eluted from hemoglobin-agarose beads.

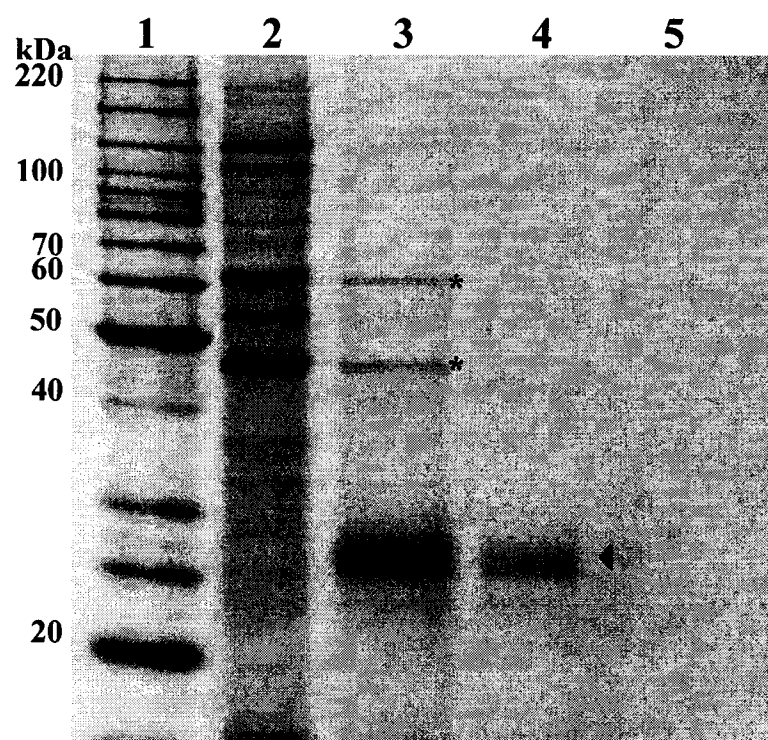
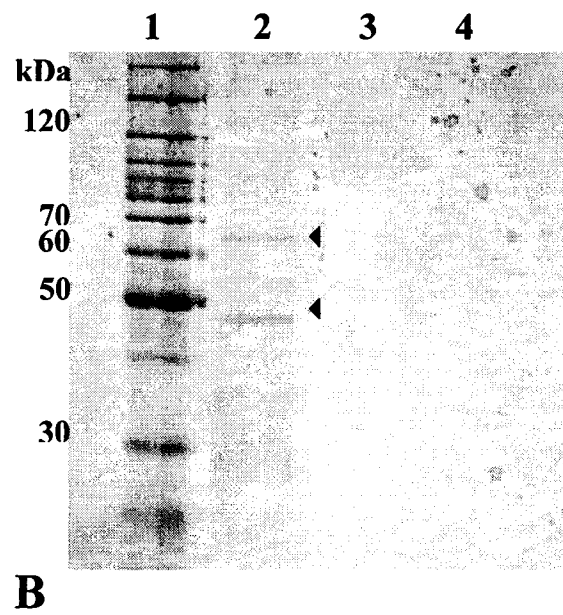
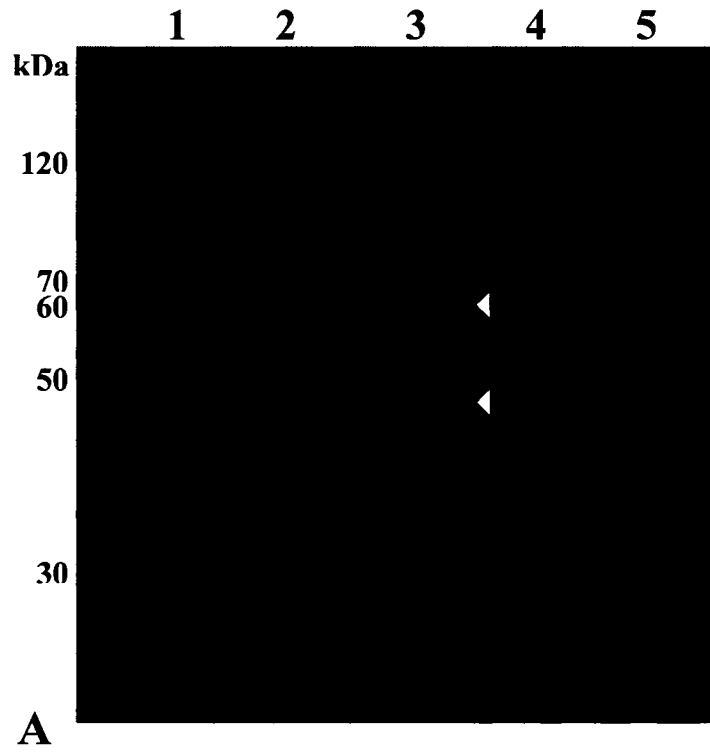


Figure 5. Identification of hemoglobin-binding proteins of *T. vaginalis* strain OC15 using streptavidin-affinity chromatography. Membrane extracts were incubated with biotinylated human hemoglobin (5 μ M)), and then incubated with immobilized streptavidin. Bound proteins were eluted from streptavidin-agarose beads with SDS-PAGE sample buffer, and analysed by SDS-PAGE (3% stacking, 10% separating gel).

- (A) An SDS-PAGE gel stained with SYPRO Ruby protein gel stain. Molecular mass protein marker in kDa (lane 1); *T. vaginalis* membrane extract (lane 2); streptavidin affinity-purified membrane proteins from *T. vaginalis* (lane 3); immobilized streptavidin control (lane 4); concentrated last wash (lane 5). Arrowheads show the position of the 48- and 63-kDa hemoglobin-binding proteins.
- (B) An SDS-PAGE gel stained with Coomassie brilliant blue. Molecular mass protein marker in kDa (lane 1); streptavidin affinity-purified membrane proteins from *T. vaginalis* (lane 2); immobilized streptavidin control (lane 3); concentrated last wash (lane 4). Arrowheads mark the 48- and 63-kDa hemoglobin-binding proteins.



These results confirmed that *T. vaginalis* is able to bind directly to hemoglobin through these two proteins, and also suggested that these proteins and hemoglobin bind strong enough to withstand several washes.

Streptavidin affinity chromatography was performed to confirm that the 48- and 63-kDa bands are hemoglobin-binding proteins. Since the protein bands purified by this technique had a lower intensity when compared to the protein bands eluted by hemoglobin-agarose beads, the other experiments in this study were carried out using hemoglobin-affinity chromatography.

3.6. Western blot analysis of proteins recovered by affinity chromatography

In order to confirm our hypothesis, which stated that the hemoglobin-binding proteins were located on the trichomonad cell surface, live trichomonads were biotin labeled before membrane extraction. Total protein extract and membrane proteins were incubated with hemoglobin-agarose beads, and the adsorbed proteins were eluted with SDS-PAGE sample buffer (without reducing agent β -mercaptoethanol).

Two biotinylated proteins of approximately 48 and 63 kDa were revealed when eluted proteins were subjected to SDS-PAGE and Western blotting with avidin-HRP (Figure 6). This result further reinforced our hypothesis that the hemoglobin-binding proteins are present on the cell surface of trichomonads, since as described in Materials and Methods, only cell surface proteins would be biotinylated under the conditions applied.

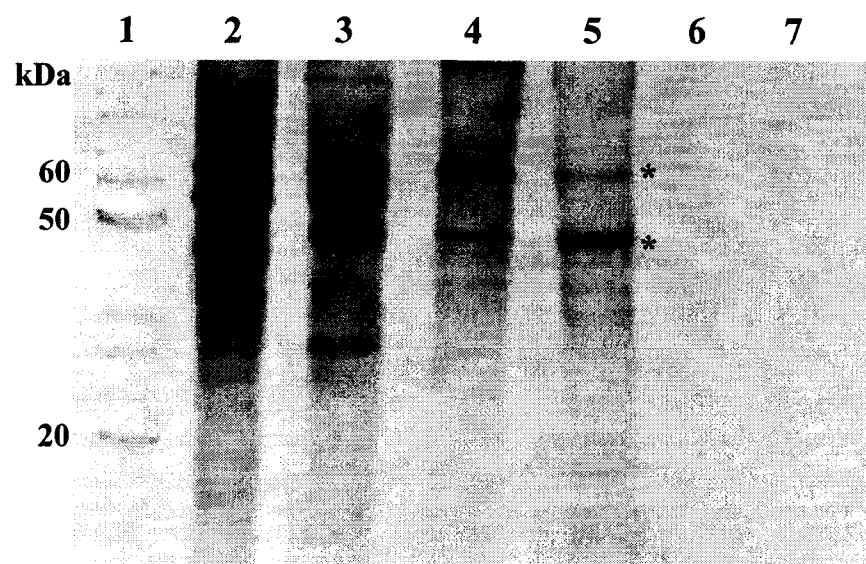
3.7. Hemoglobin-binding proteins in trichomonads grown in various iron concentrations

In order to determine whether the expression of these two hemoglobin-binding proteins is regulated by iron, trichomonads were grown in different iron concentrations as

Figure 6. Western blot analysis of hemoglobin-binding proteins isolated by affinity chromatography. Live trichomonads were biotin-labeled, and the membrane proteins were extracted and incubated with hemoglobin-agarose beads. The eluted protein from hemoglobin-agarose beads were subjected to SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and stained with avidin-HRP.

Molecular mass protein marker in kDa (lane 1); biotinylated total protein extract from *T. vaginalis* (lane 2); biotinylated membrane proteins (lane 3); hemoglobin-agarose affinity-purified total protein extract (lane 4); hemoglobin-agarose affinity-purified membrane proteins (lane 5); hemoglobin-agarose control (lane 6); concentrated last wash (lane 7).

Asterisks indicate the position of the 48- and 63-kDa biotinylated hemoglobin-binding proteins.



described in Materials and Methods. Membrane fractions from trichomonads grown in low-iron, high-iron, and hemoglobin-added TYI as well as membrane proteins of trichomonads grown in normal conditions were subjected to affinity purification with hemoglobin-agarose beads. SDS-PAGE analysis of the eluted proteins showed no detectable differences in the gel profiles, and the same two hemoglobin-binding protein bands that had been detected in normal conditions were revealed in other samples with the same intensity regardless of the iron levels of the growth medium (Figure 7).

This result indicated that the expression of hemoglobin-binding proteins appear unchanged under different concentrations of iron, and suggesting that the hemoglobin-binding property is a constitutive factor in *T. vaginalis*, and is iron-independent.

3.8. Competition binding assay

In order to determine whether these two proteins bind specifically to hemoglobin, competition analysis was carried out. For doing this, membrane proteins were pre-incubated with 1mM hemoglobin or hemin for one hour before incubation with hemoglobin-agarose beads. In some experiments 1mM hemoglobin or hemin was co-incubated with membrane proteins and hemoglobin-agarose beads. In both sets of experiments the results were the same: hemoglobin, but not hemin could completely block the 63-kDa band, indicating the specificity of this binding protein to hemoglobin and not hemin. The 48-kDa protein was partially inhibited by excess hemoglobin, however, was completely inhibited in the presence of heme. This result suggested that the 48-kDa protein may recognize and bind to hemoglobin via the heme moiety (Figure 8).

The same result was obtained when membrane extracts of trichomonads grown in low- and high-iron conditions were pre- or co-incubated with 1mM hemoglobin or 1mM

Figure 7. Hemoglobin-binding proteins purified by affinity chromatography from membrane fractions of *T. vaginalis* grown under various iron concentrations. SDS-PAGE gel stained with RAPID stain. Expression of hemoglobin-binding proteins remained unchanged under different concentrations of iron in the culture medium.

Molecular mass protein marker in kDa (lane 1); *T. vaginalis* grown in normal medium membrane extract (lane 2); hemoglobin-agarose affinity-purified membrane proteins from *T. vaginalis* grown in normal medium (lane 3); membrane extract from trichomonads grown under low-iron conditions (lane 4); hemoglobin-agarose affinity-purified membrane proteins from trichomonads grown under low-iron conditions (lane 5); membrane extract from trichomonads grown in high-iron conditions (lane 6); hemoglobin-agarose affinity-purified membrane proteins from trichomonads grown in high-iron conditions (lane 7); membrane extract from trichomonads grown in hemoglobin-added TYI (lane 8); hemoglobin-agarose affinity-purified membrane proteins from trichomonads grown in hemoglobin-added TYI (lane 9).

Asterisks indicate the position of the 48- and 63-kDa hemoglobin-binding proteins. Arrowhead indicates the hemoglobin eluted from hemoglobin-agarose beads.

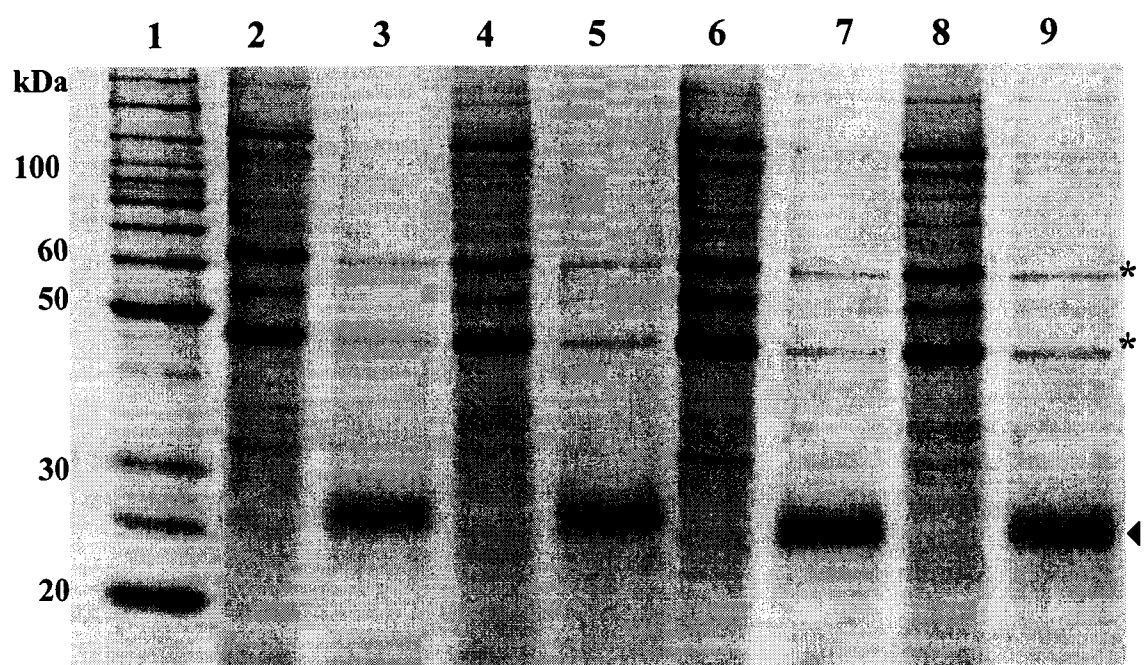
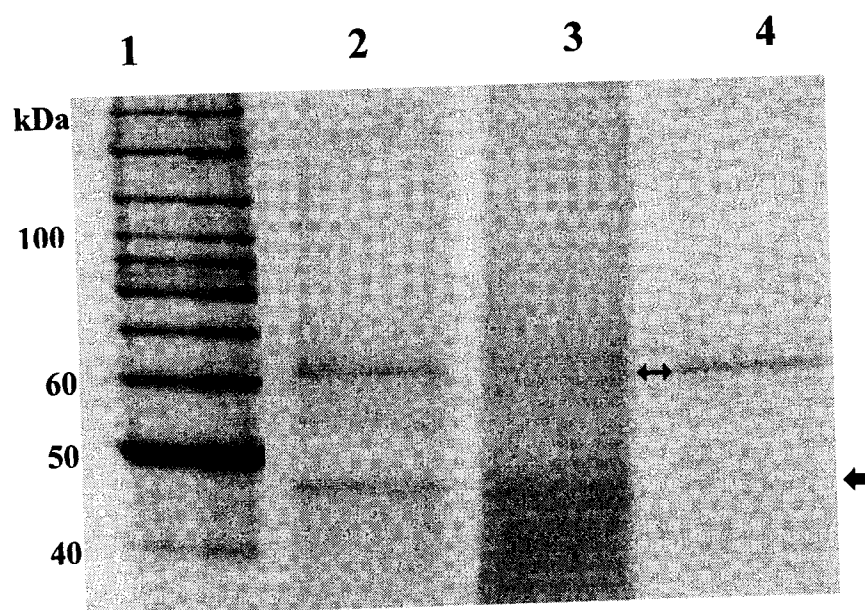


Figure 8. Competition binding assay with excess human hemoglobin or bovine hemin solutions in hemoglobin-affinity chromatography. Membrane proteins of *T. vaginalis* grown in normal medium were pre- or co-incubated with 1 mM human hemoglobin or 1mM bovine hemin solutions. The eluted proteins were analysed by SDS-PAGE. Gel stained with RAPID stain.

Molecular mass protein marker in kDa (lane 1); hemoglobin-agarose affinity-purified membrane proteins from *T. vaginalis* grown in normal medium (lane 2); *T. vaginalis* (grown in normal medium) membrane fractions pre-incubated with 1mM human hemoglobin solution (lane 3); *T. vaginalis* (grown in normal medium) membrane fractions pre-incubated with 1mM bovine hemin solution (lane 4).

The two-headed arrow shows the 63-kDa protein has been completely blocked with excess hemoglobin, but not with hemin. The arrow indicates how the 48-kDa protein has been partially inhibited by excess hemoglobin, whereas hemin solution could completely inhibit the protein band.



hemin before purification by hemoglobin-affinity chromatography (Figure 9), corroborated one more time that the 63-kDa protein is specific for hemoglobin binding, and the 48-kDa protein should be considered primarily as a heme-binding protein.

Incubation of *T. vaginalis* membrane proteins with different human hemoglobin concentrations (from 10 μ M to 1mM) before or during affinity chromatography with hemoglobin-agarose beads, demonstrated that excess hemoglobin could block the 63-kDa protein in a concentration-dependent manner (Figure 10).

3.9. Heme-affinity chromatography

To confirm that the 48-kDa protein specifically binds to hemin, heme-agarose beads were used in an affinity chromatography study to determine whether they could purify the same 48-kDa protein band. Since all *T. vaginalis* cells, regardless of the iron concentrations in their growth medium, were able to express this band, trichomonads grown in normal medium was used for this set of experiments. Membrane proteins were then subjected to affinity purification with hemin-agarose beads, and purified proteins were eluted with 2 x SDS-sample buffer, and were analysed with SDS-PAGE. As expected, a 48-kDa protein band was identified with this method, supported the conclusion that this protein is more specific for heme and should be considered as a heme-binding protein (Figure 11).

Hemin-agarose beads could also adsorb the 63-kDa protein but in less intensity comparing to hemoglobin-agarose beads. No specific bands were observed in hemin-agarose beads control and in concentrated last wash (Figure 11).

Competition assay, in which membrane proteins were pre- or co-incubated with 100 μ M human hemoglobin or 100 μ M bovine hemin solutions, showed that excess hemin could

Figure 9. Competition binding assay with excess human hemoglobin or bovine hemin solutions. Membrane proteins of *T. vaginalis* grown in low- and high-iron media were pre- or co-incubated with 1 mM human hemoglobin or 1mM bovine hemin solutions. The eluted proteins were analysed by SDS-PAGE. Gel stained with RAPID stain.

Molecular mass protein marker in kDa (lane 1); hemoglobin-agarose affinity-purified membrane proteins from *T. vaginalis* grown in high-iron medium (lane 2); *T. vaginalis* (grown in high-iron medium) membrane fractions pre-incubated with 1mM human hemoglobin solution (lane 3); *T. vaginalis* (grown in high-iron medium) membrane fractions pre-incubated with 1mM bovine hemin solution (lane 4); hemoglobin-agarose affinity-purified membrane proteins from *T. vaginalis* grown in low-iron medium (lane 5); *T. vaginalis* (grown in low-iron medium) membrane fractions pre-incubated with 1mM human hemoglobin solution (lane 6); trichomonad (grown in low-iron medium) membrane fractions pre-incubated with 1mM bovine hemin solution (lane 7).

The two-headed arrows show the 63-kDa protein bands have been completely blocked with excess hemoglobin, but not with hemin. The asterisks indicate how the 48-kDa proteins have been partially inhibited by excess hemoglobin, whereas the arrowheads show that hemin solution could completely inhibit the protein bands.

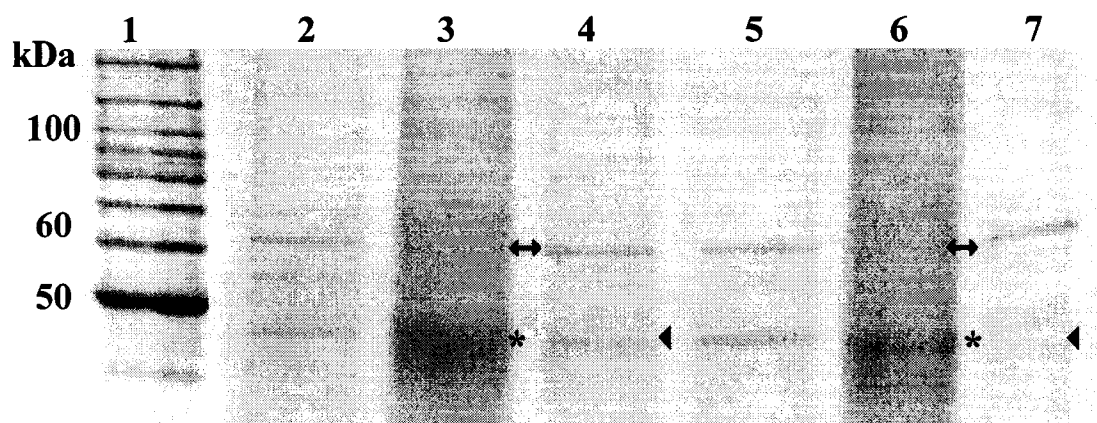


Figure 10. Competition binding assay with different concentrations of human hemoglobin solution. Membrane proteins of *T. vaginalis* grown in normal medium were pre- or co-incubated with different concentrations of human hemoglobin (from 10 μ M to 1 mM). The eluted proteins were analysed by SDS-PAGE. Gel stained with RAPID stain.

Molecular mass protein marker in kDa (lane 1); membrane proteins of *T. vaginalis* (lane 2); hemoglobin-agarose affinity-purified membrane proteins (lane 3); trichomonad membrane fractions pre-incubated with 10 μ M human hemoglobin solution (lane 4); trichomonad membrane fractions pre-incubated with 50 μ M human hemoglobin solution (lane 5); trichomonad membrane fractions pre-incubated with 100 μ M human hemoglobin solution (lane 6); trichomonad membrane fractions pre-incubated with 1 mM human hemoglobin solution (lane 7).

The asterisk shows the 63-kDa protein is inhibited by excess hemoglobin in a concentration-dependent manner.

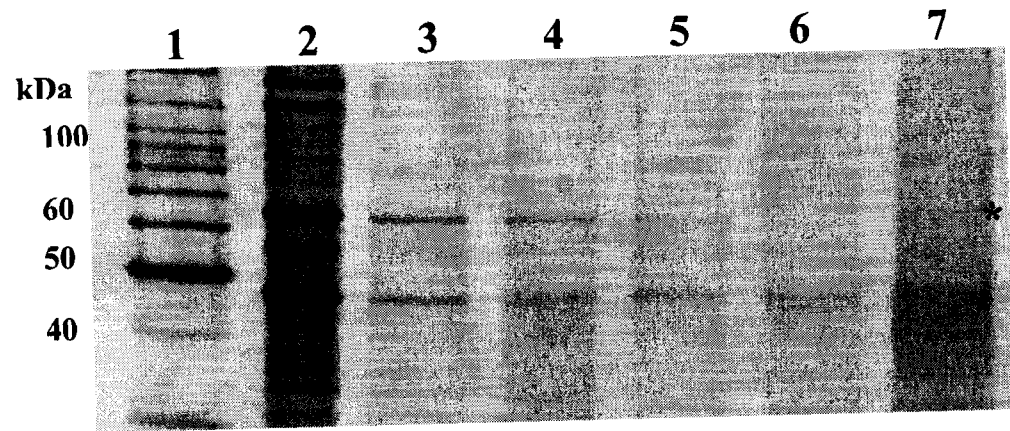
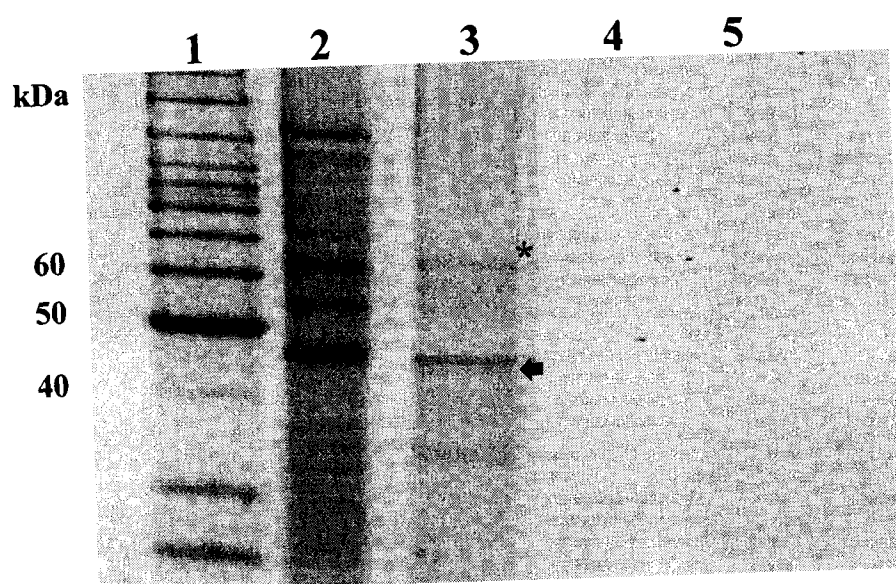


Figure 11. Identification of heme- and hemoglobin-binding proteins of *T. vaginalis* using Heme-affinity chromatography. Membrane proteins of *T. vaginalis* grown in normal medium incubated with hemin-agarose beads. The eluted proteins were analysed by SDS-PAGE. Gel stained with RAPID stain.

Molecular mass protein marker in kDa (lane 1); membrane proteins of *T. vaginalis* (lane 2); heme-agarose affinity-purified membrane proteins from *T. vaginalis* (lane 3); hemin-agarose beads control (lane 4); concentrated last wash (lane 5).

The arrow shows the 48-kDa protein, and the asterisk indicates the 63-kDa protein.



totally inhibit the 48-kDa protein band, whereas hemoglobin could partially block this band (Figure 12). This result indicated one more time that the heme portion of hemoglobin is recognized by this binding protein.

3.10. Western blot analysis using biotinylated-hemoglobin or anti-human hemoglobin antiserum

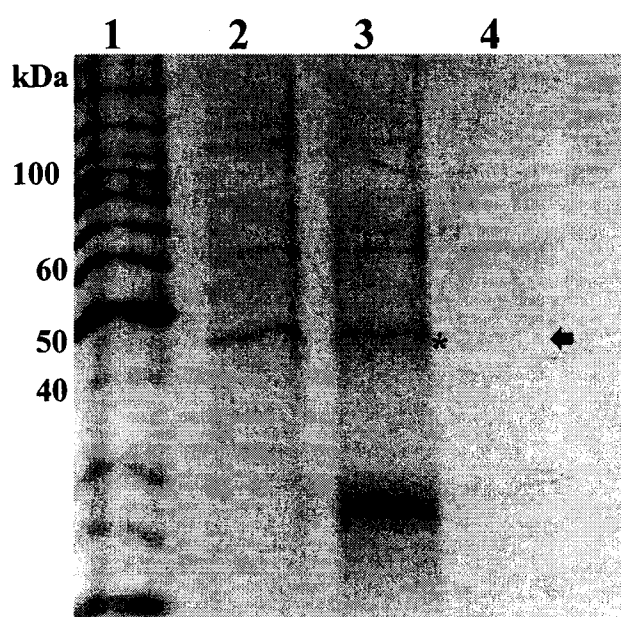
T. vaginalis total proteins obtained from trichloroacetic acid (TCA) precipitation method along with membrane proteins were subjected to SDS-PAGE and a duplicate gel was transferred onto polyvinylidene fluoride (PVDF) membrane. After blocking, the membrane was incubated with either biotinylated-human hemoglobin or human hemoglobin solution. Later avidin-HRP or antiserum against human hemoglobin was used to detect the hemoglobin-binding proteins. However, neither of these two protocols provided any result to support our previous findings with affinity chromatography (data not shown). Since these protocols were already used successfully in dot blot assay (Figures 2 and 3), the recent results suggested that the procedures in which the protein samples were prepared in SDS-sample buffer and separated by SDS-PAGE could affect the binding activity of these proteins, due to the changes in their structure. From these results, it is indicated that the 48- and 63-kDa proteins need their intact tertiary structure in order to functionally bind to heme or hemoglobin.

Nondenaturing SDS-PAGE (native gel) could not reveal these two bands either. Due to the nature of a native gel, proteins bands were clumped together, and made it difficult to identify the desired bands (data not shown).

Figure 12. Competition binding assay with excess human hemoglobin or bovine hemin solutions, using heme-affinity chromatography. Membrane proteins of *T. vaginalis* grown in normal medium incubated with 100 μ M heme or human hemoglobin solutions before subjected to affinity purification with heme-agarose beads. The eluted proteins were analysed by SDS-PAGE. Gel stained with RAPID stain.

Molecular mass protein marker in kDa (lane 1); heme-agarose affinity-purified membrane proteins from *T. vaginalis* (lane 2); membrane proteins pre-incubated with 100 μ M human hemoglobin solution (lane 3); membrane fractions pre-incubated with 100 μ M bovine hemin solution (lane 4).

The asterisk indicates the 48-kDa protein, which was not blocked by adding excess hemoglobin, and the arrow shows how pre-incubation with hemin could compete this band out.



3.11. Mass spectral analysis of the 48- and 63-kDa proteins

To identify the 48- and 63-kDa hemoglobin-binding proteins isolated by hemoglobin-affinity chromatography, these protein bands were excised from the Coomassie blue stained polyacrylamide gel, trypsin digested, and analysed by MALDI-TOF mass spectrometry. The mass spectrometry results were then searched against the protein databases using the Mascot search engine. The Mascot search results were summarized in Table 2 and 3.

Peptides identified by Mascot were also analysed with BLAST search program of NCBI, and confirmed that the 48-kDa protein has 100% identity to subunits of *T. vaginalis* adhesin protein AP51 (AP51-3, AP51-2, and AP51-1), and Succinyl-CoA ligase beta-chain, hydrogenosomal precursor (*T. vaginalis*). The 63-kDa protein also showed 100% identity to subunits of *T. vaginalis* adhesin protein AP65 (AP65-3, AP65-2, and AP65-1), adhesin AP65-1 precursor (*T. vaginalis*), malate dehydrogenase: subunit A (*T. vaginalis*), hydrogenosomal malic enzyme subunit D proprotein (*T. vaginalis*), hydrogenosomal malic enzyme subunit C proprotein (*T. vaginalis*), and hydrogenosomal malic enzyme subunit B proprotein.

3.12. Hemoglobin-binding activity in trichomonads with silenced *ap65-3* gene

Sequencing analysis revealed that the 48- and 63-kDa proteins have significant identities to *T. vaginalis* adhesin subunits AP51 and AP65, respectively. In order to confirm the AP65 role in binding to hemoglobin, hemoglobin-binding activity of trichomonads with silenced *ap65* gene (AS or antisense transfectants) was studied and compared to the same activity in their wild type (isolate T016), and sens (S) transfectants. T016 isolate, and both S and AS transfectants were kindly supplied by Dr. John F. Alderete. It had been already showed that antisense silencing of AP65-3 gene resulted in decreased amount of AP65,

Table 2. Result from mass spectrometry of the 48-kDa hemoglobin-binding protein. The 48-kDa band was excised from a SDS-PAGE gel followed by in-gel trypsin digestion. Further analysis by mass spectrometry in conjunction with Mascot search of sequence databases indicated that this protein has significant homology with *T. vaginalis* adhesin protein AP51 (subunit 3), and Succinyl-CoA ligase [GDP-forming] beta-chain, hydrogenosomal precursor (Succinyl-CoA synthetase, beta-chain).

A Mascot score over 53 was considered a significant match between a database sequence search and the identified sequences from mass spectrometry ($p < 0.05$).

Adhesin protein AP51-3 (*T. vaginalis*)

Mass: 43732 Score: 316 Queries matched: 4

Score	Peptide
74	AILVNIFGGIVR
95	AILVNIFGGIVR
91	LFVGSDATQVEVNPLAETTDGR
132	LAGGDPANFLDLGGAASEAAVTEGFTIISQSHVK

**Succinyl-CoA ligase [GDP-forming] beta-chain,
hydrogenosomal precursor (Succinyl-CoA synthetase,
beta-chain)**

Mass: 44087 Score: 266 Queries matched: 4

Score	Peptide
86	YNVAAGINLVAR
74	AILVNIFGGIVR
95	AILVNIFGGIVR
91	LFVGSDATQVEVNPLAETTDGR

Table 3. Result from mass spectrometry of the 63-kDa hemoglobin-binding protein. The 63-kDa band was excised from gel, trypsin digested and further characterized by mass spectrometry. Mascot search of sequence databases indicated that this protein has homology with *T. vaginalis* hydrogenosomal malic enzyme subunit B proprotein precursor, adhesin protein AP65-2, and AP65-1.

A Mascot score over 55 was indicated identity or extensive homology ($p < 0.05$).

Hydrogenosomal malic enzyme subunit B proprotein precursor (<i>T. vaginalis</i>) Mass: 63636 Score: 409 Queries matched: 4	
Score	Peptide
93	TGMTLLQDGDLSK
117	YLIDNVLATAVPPK
102	ALCATGSPFPAEQVNGR
101	EIVQATLENAEHPVIMPLSNPTPK
AP65-2 adhesin (<i>T. vaginalis</i>) Mass: 63618 Score: 192 Queries matched: 2	
Score	Peptide
93	TGMTLLQDGDLSK
99	ATSVIGVSGVPGLITK
AP65-1 adhesin (<i>T. vaginalis</i>) Mass: 63698 Score: 189 Queries matched: 2	
Score	Peptide
87	TGMTLLQDGDLNK
102	ALCATGSPFPAEQVNGR

which was subsequently related to lower levels of adherence to the vaginal epithelial cells, when compared to the wild type or S transfectants (Mundodi, et al., 2004).

AS and S transfecting *T. vaginalis*, in addition to the wild type trichomonads (T016 isolate) were grown under normal iron concentration. Membrane proteins were extracted and subjected to hemoglobin-affinity chromatography. Purified proteins were separated by SDS-PAGE (Figure 13). As expected, the intensity of the 63-kDa protein band was weaker in AS transfectants comparing to the same band in S transfectants, wild type, and OC15 isolate (Figure 13). Total protein extracts of *T. vaginalis* isolate T016, S and AS transfectants were also purified with hemoglobin-affinity chromatography. This result also indicated that the 63-kDa protein band in AS transfecting trichomonads appeared weaker in intensity when it compared to the same band purified from the wild type or S transfecting total protein extracts (Figure 14).

3.13. Hemoglobin-binding activity in DG isolate

As discussed earlier, DG isolates have lost its pathogenicity due to prolonged passaging. These trichomonads no longer cause subcutaneous abscess nor vaginitis in our lab mouse model. However, as demonstrated earlier in this project, hemolytic activity of these trichomonads did not display any significant difference from other *T. vaginalis* isolates. Hemoglobin-binding activity of this isolate was also studied. When membrane extracts of DG trichomonads grown in normal iron conditions were subjected to affinity chromatography with hemoglobin-agarose beads, the same 46- and 63-kDa proteins were identified (Figure 15). This result showed that other than having the same hemolytic activity as the pathogenic isolate of *T. vaginalis*, this isolate is also able to bind and utilize hemoglobin as a source of iron.

Figure 13. Hemoglobin-affinity chromatography of wild type and transfected trichomonads. *T. vaginalis* (isolate OC15) along with wild type, S and AS transfected *T. vaginalis* (isolate T016) were grown under normal iron concentration. Membrane proteins were purified with affinity chromatography and visualized by SDS-PAGE. Gel was stained with RAPID stain. The intensity of the 63-kDa protein band was weaker in AS transfectants comparing to the same band in S transfectants, wild type, and OC15 isolate

Molecular mass protein marker in kDa (lane 1); membrane extraction of *T. vaginalis* (isolate OC15) (lane 2); hemoglobin-agarose affinity-purified membrane proteins from *T. vaginalis* (OC15) (lane 3); membrane proteins of the wild type trichomonads (T016) (lane 4); hemoglobin-agarose affinity-purified membrane proteins from wild type trichomonads (T016) (lane 5); membrane extraction of S transfected trichomonads (lane 6); hemoglobin-agarose affinity-purified membrane proteins from S transfected trichomonads (lane 7); membrane extraction of AS transfected trichomonads (lane 8); hemoglobin-agarose affinity-purified membrane proteins from AS transfected trichomonads (lane 9).

Asterisk indicates 63-kDa hemoglobin-binding. Arrowhead indicates the hemoglobin eluted from hemoglobin-agarose beads.

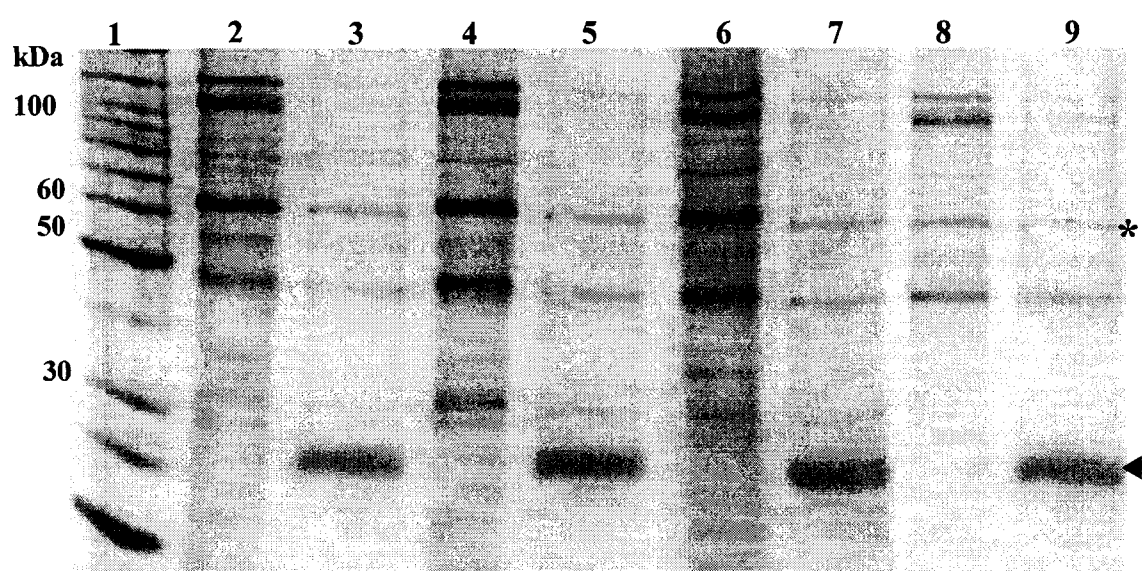


Figure 14. Hemoglobin-affinity chromatography of total protein preparations of wild type, S and AS transfected *T. vaginalis* (T016). Trichomonads were grown under normal iron concentration. SDS-PAGE gel was stained with RAPID stain.

Molecular mass protein marker in kDa (lane 1); total proteins of the wild type trichomonads (lane 2); hemoglobin-agarose affinity-purified proteins from wild type trichomonads (lane 3); total proteins of S transfected trichomonads (lane 4); hemoglobin-agarose affinity-purified total proteins from S transfected trichomonads (lane 5); total proteins of AS transfected trichomonads (lane 6); hemoglobin-agarose affinity-purified total proteins from AS transfected trichomonads (lane 7).

Asterisk indicates that the intensity of the 63-kDa protein band was weaker in AS transfectants comparing to the same band in S transfectants, and wild type. Arrowhead indicates the hemoglobin eluted from hemoglobin-agarose beads.

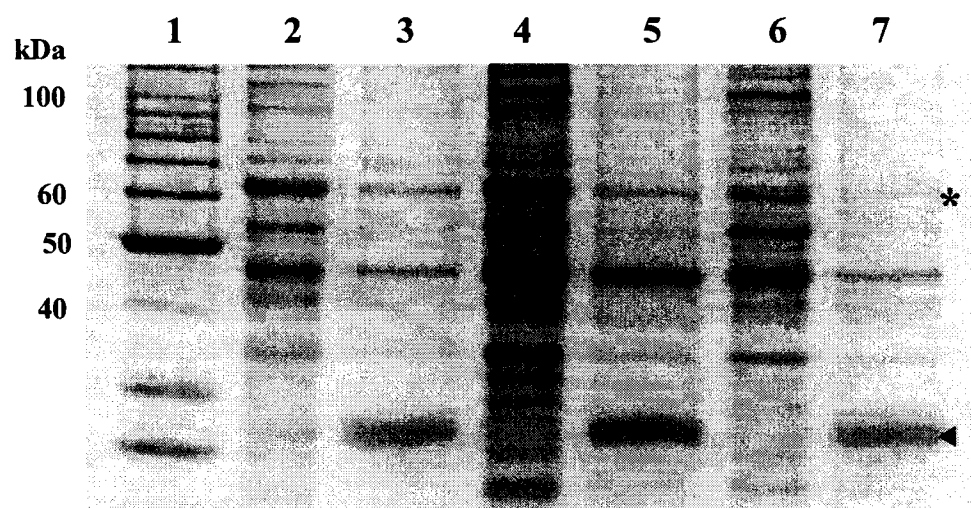
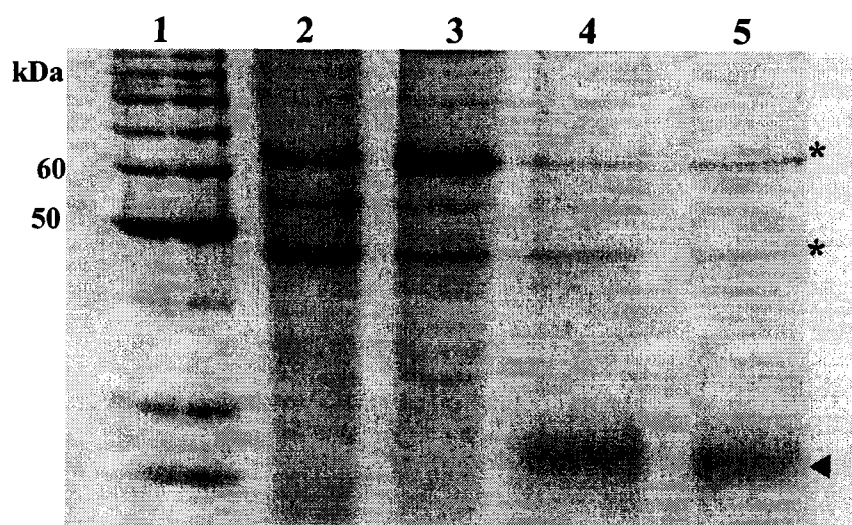


Figure 15. Hemoglobin-affinity chromatography of membrane extractions of *T.vaginalis* (isolates OC15 and DG) showed no difference in the ability to bind hemoglobin between these two isolates. SDS-PAGE gel was stained with RAPID stain.

Molecular mass protein marker in kDa (lane 1); membrane proteins of *T.vaginalis* (isolate OC15) (lane 2); membrane proteins of *T.vaginalis* (isolate DG) (lane 3); hemoglobin-agarose affinity-purified membrane proteins of *T.vaginalis* (isolate OC 15) (lane 4); hemoglobin-agarose affinity-purified membrane proteins of *T.vaginalis* (isolate DG) (lane 5).

Asterisks indicate the position of the 48- and 63-kDa hemoglobin-binding proteins in both isolates. Arrowhead indicates the hemoglobin eluted from hemoglobin-agarose beads.



CHAPTER FOUR: DISCUSSION

4.1. Hemolytic activity of *T. vaginalis* isolates

It has been nearly half a century since trichomoniasis was established as a sexually transmitted disease. However, it was not considered as an important STD until recently when the association of *T. vaginalis* infection with adverse pregnancy outcomes and increasing risk of HIV transmission were shown (Carr, et al., 1998; Paterson et al, 1998).

Little is known about pathogenicity of *T. vaginalis*. It has been demonstrated that this parasite like several other protozoan parasites including *Trypanosoma congolense*, *T. brucei*, and *Entamoeba histolytica* has a hemolytic activity (Tasca and de Carli, 1999). The importance of this activity in the host-parasite relationship is not clearly understood. However, it has been suggested that since *T. vaginalis* is deficient in major biosynthetic pathways, lysing erythrocytes can be beneficial to the parasite by providing essential nutrients such as lipids and iron (Dailey et al, 1990). Krieger and his group (1983) suggested that hemolytic activity in *T. vaginalis* might be related to virulence. In our study, all four isolates, whether from asymptomatic patients or from patients with vaginitis, were capable of lysing red blood cells, and no significant difference between hemolytic activity in different pathogenic *T. vaginalis* isolates was observed ($P<0.05$). This discrepancy may be due to the different experimental preparations, or to the differences between the *T. vaginalis* strains used in these two studies. All four trichomonal isolates in our study, regardless of whether grown for short or long periods of time (OC15 isolate versus DG isolate) had the ability to lyse sheep red blood cells. Therefore, prolonged cultivation in axenic growth medium does not affect trichomonal hemolytic activity, although it does affect in vivo mouse model infectivity.

Based on our results, we concluded that although hemolytic activity has an important role in the growth and survival of *T. vaginalis* in a nutrient-limited environment, this activity alone cannot be considered as a virulence marker for trichomoniasis.

4.2. Heme- and hemoglobin-binding proteins in *T. vaginalis*

Iron is an essential nutrient for *T. vaginalis*. Although the human body has an abundant source of iron, the availability of free iron for pathogenic microorganisms is strictly limited. Ferric iron is sequestered by the host iron-binding glycoproteins transferrin and lactoferrin, and in the blood, most iron is in erythrocytic hemoglobin (Frangipane et al., 1994). The iron requirement of *T. vaginalis* can be satisfied by phagocytosis of erythrocytes and vaginal epithelial cells (Rendón-Maldonado et al., 1998). *T. vaginalis* can bind to lactoferrin, which is found in cervical secretions, through a specific receptor, and use it as an iron source (Peterson and Alderete, 1984). Hemoglobin can also serve as an alternative source of heme-iron for this parasitic protozoan (Lehker et al., 1990). It has been already demonstrated that trichomonads were able to grow in medium supplemented with hemoglobin or hemin, as the only source of iron (Alderete, et al., 2004). It has also been suggested that hemoglobin released from erythrocytes, bind to the parasites in a specific, receptor-mediated manner (Lehker et al., 1990). Therefore, our main goal in this project was to determine whether *T. vaginalis* was able to bind hemoglobin through specific binding proteins, as a possible initial step in the acquisition of heme-iron from hemoglobin.

In order to answer this question, the first step was to confirm previously published findings on hemoglobin-binding activity of *T. vaginalis*. OC15, which is a pathogenic human trichomonad isolate, was grown in regular iron conditions, and the membrane extractions were prepared by gentle homogenization. The ability of this parasite to bind to

hemoglobin was demonstrated by dot blot assay, using biotinylated hemoglobin as a probe. It was also shown that trichomonal membrane extracts were able to bind to biotinylated hemoglobin in a concentration-dependant manner. The same result was obtained when dot-blotted membrane proteins were incubated with human hemoglobin solution and hemoglobin-binding activity of trichomonads was detected in the presence of anti-human hemoglobin antibody. These results reaffirm that hemoglobin-binding activity exists in this pathogenic protozoan.

The next step was to identify the hemoglobin-binding protein(s). Two protein bands with the molecular weight of approximately 48 and 63 kDa were revealed when *T. vaginalis* membrane extracts were subjected to affinity purification assay using immobilized hemoglobin or streptavidin on agarose as the ligand. Hemoglobin binds to these two protein bands with high affinity, since no bands were detected in concentrated final washes.

To confirm that these hemoglobin-binding proteins are localized on *T. vaginalis* membrane, cell surface proteins were labeled with biotin, as described previously. Western blotting with avidin-HRP demonstrated that these 48- and 63-kDa proteins are membrane proteins.

In order to investigate whether the expression of these two proteins was regulated by iron, trichomonads of *T. vaginalis* isolate OC15 were grown under different concentrations of iron. It was also of interest to find out whether trichomonads grown under iron-depleted condition could express other hemoglobin-binding protein(s). Trichomonads grown under low-iron condition showed different growth rate depending on the concentration of FBS, which contains between 1.2 to 1.8 mg/ml of iron-binding glycoprotein transferrin (Gifford et al., 2002), added to the medium. Although it has been reported that *T. vaginalis* does not have a receptor for transferrin (Lehker et al., 1990), it has been suggested that trichomonads

are able to acquire iron from transferrin by secreting acidic low-molecular weight substances into their environment (Nelson, 2000). Acidification of the environment may subsequently result in loss of iron from transferrin, which was later transported into the trichomonad cytoplasm, and ultimately to the hydrogenosomes (Nelson, 2000). Our results also suggest that trichomonads are able to take up iron from transferrin in FBS, and FBS can be considered as an important source of iron especially for *T. vaginalis* isolates grown in low-iron axenic culture conditions. To maintain low-iron conditions, the amount of FBS added to *T. vaginalis* growth medium was decreased to 2.5% (trichomonal growth rate was significantly decreased by FBS amount less than 2.5%). The same condition was applied to trichomonads grown in hemoglobin-supplemented TYI. Dot blot analysis did not reveal any differences in hemoglobin-binding activity of trichomonads grown in normal growth conditions compared with those grown under iron-deficient and iron-rich conditions. Similarly, SDS-PAGE analysis of purified proteins from hemoglobin affinity chromatography did not show any noticeable difference in intensity of the two protein bands between parasites grown under different iron concentrations. No other protein band was also detected in membrane extractions of trichomonads grown under iron-depleted conditions. These results suggest that the synthesis of hemoglobin-binding proteins in *T. vaginalis* is not iron-regulated. Similar results have also been reported in other pathogenic microorganisms (Guan et al., 2002; Mazoy et al., 1996). It appears that the ability to bind hemoglobin is an important factor for *T. vaginalis* growth and survival, and these binding proteins are constitutively expressed at a basal level, irrespective of the iron concentration in the growth medium.

Prolonged cultivation in axenic growth medium also does not affect the expression of these hemoglobin-binding proteins, since the DG isolate, which is a long-term

laboratory isolate, demonstrated the 48- and 63-kDa protein bands with the same intensity as observed in the OC15 isolate.

The competitive binding assay using excess hemoglobin solution showed that hemoglobin could block the 63-kDa band in a concentration-dependent manner. However the 48-kDa band was only partially inhibited when membrane extracts were incubated with excess hemoglobin prior to affinity purification. Moreover, when excess heme solution was used in a competition assay instead of hemoglobin, heme could completely block the 48-kDa band, but has no blocking effect on the 63-kDa band. These findings suggested that the 48-kDa hemoglobin-binding protein has a higher affinity for heme, and therefore binds to the heme moiety of hemoglobin. Affinity chromatography on a heme-agarose column confirmed this observation and showed that the 48-kDa protein binds to heme with higher affinity when compared to the 63-kDa protein band.

To identify the heme- and hemoglobin-binding proteins, MALDI-TOF mass spectrometry was performed. A BLAST search revealed that the 48- and 63-kDa proteins are 100% identical to the subunits of *T. vaginalis* adhesin proteins AP51 and AP65, respectively. Therefore, It was not surprising that AS transfected trichomonads, in which the expression of *ap65* gene was reduced, showed lower hemoglobin-binding activity when compared with the same activity in the wild type and S transfectants.

Vaginal epithelial cells, which are the main target cells for *T. vaginalis*, mature under hormonal control during the progression of the menstrual cycle. These cells may express different receptors during their maturation process and menstrual cycle. Therefore, a pattern more complex than a "one adhesin / one receptor" paradigm is needed to allow *T. vaginalis* to survive in the vagina throughout the menstrual cycle. The presence of multiple adhesins may be advantageous to the parasite existing in an ever-changing host environment (Alderete

et al., 1995a). This complex interaction between trichomonads and target cells is specifically regulated by iron, and maximal levels of cytoadherence and increased expression of four trichomonad adhesins occurs when the parasites are grown in iron-supplemented medium (Lehker et al., 1991). However, it has been noted that low-iron grown parasites express a basal level of adhesins (Alderete et al., 1995a). Other than vaginal epithelial cells, it has been reported that all four *T. vaginalis* adhesins (AP65, AP51, AP33, and AP23) are able to bind to erythrocytes (Addis et al., 2000). The AP65, which appears to be a major adhesin, not only has the ability to adhere to its target cells (erythrocytes and epithelial cells), but is also secreted by trichomonads both in vitro and in vivo (Rappelli, et al., 1995). It has been suggested that AP65 is first exposed on the trichomonal cell surface for a period of time before releasing to the extracellular environment (Addis et al., 1997).

According to our experimental results and what other investigators have observed, it is hypothesized that during menstruation when the amount of lactoferrin (a key iron source for *T. vaginalis*) is low, trichomonads are under low-iron conditions. This situation resulted in downregulation of adhesins synthesis, and decrease cytoadherence to the epithelial cells. However, because of the basal level of adhesins (specifically AP65 and AP51), and the secreted AP65 in the environment, trichomonads are able to bind erythrocytes, which are abundant during menstruation. Shortly after binding, hemolysis occurs, and free hemoglobin is released, and immediately binds to trichomonads through their hemoglobin-binding proteins (AP65 and AP51). Hemoglobin can be phagocytosed by trichomonads, or be degraded into heme and globin by a *T. vaginalis* 60-kDa cysteine proteinase (Min et al., 1998). Heme-iron can bind to AP51, and then be transported into the cytoplasm, causing an increase in the internal iron pools of *T. vaginalis*. Increased amount of iron leads to an increase in growth and multiplication rates of the parasite, in addition to

the higher expression of adhesins on the cell surface and therefore, elevated trichomonal adherence to the vaginal epithelial cells, post menses.

The above hypothetical model emphasizes the important role that iron plays in regulation of adhesins as a mechanism that enables the parasite to adapt to the complex and constantly changing environment of the vagina during the menstrual cycle.

The BLAST search also showed that the 48- and 63-kDa proteins have equal identity to *T. vaginalis* hydrogenosomal enzymes. Hydrogenosomes are double membrane-bound organelles which metabolize pyruvate using pyruvate:ferredoxin oxidoreductase (PFO), and produce hydrogen through the activity of hydrogenase (Müller, 1997).

This result was not surprising since it has been already reported that three of four described trichomonal adhesins (AP65, AP51, and AP33) share identity with the hydrogenosome proteins: malic enzymes, and the β - and α -subunits of Succinyl-coenzyme A synthetase (SCS), respectively (O'Brien et al., 1996; Brugerolle et al., 2000). Malic enzyme found in hydrogenosomes normally functions as a metabolic enzyme catalyzing the oxidative carboxylation of malate to pyruvate (Markos et al., 1993). SCS is another hydrogenosomal enzyme, which catalyzes the formation of ATP via substrate-level phosphorylation (Lahti et al., 1994). Multigene families encode these metabolic enzymes and their homologue adhesins (Garcia et al., 2003). It has been suggested that the hydrogenosomal enzymes, like adhesins, may be upregulated by iron (Alderete et al., 1998). It seems that these proteins are bifunctional, they work as metabolic enzymes when located on the hydrogenosomes, and act as adhesins when placed on the surface of trichomoands.

Recent studies demonstrated that *T. vaginalis* is part of a growing list of microbial pathogens that possess surface-associated enzymes (SAEs) with alternative, non-enzymatic functions (Alderete et al., 2001). Some investigators have described metabolic enzymes on

microbial surface with multiple functions, including adherence (Pancholi and Fischetti, 1992; Camara et al., 1994; Joe et al., 1994). These housekeeping enzymes, released or surface-bound, often appear to perform specific functions in the microorganism's pathogenicity. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is an enzyme with a number of functions, has been found on the surface of group A streptococci where it functions as a plasmin receptor (Lottenberg et al., 1992; Pancholi and Fischetti, 1992). This enzyme has also been reported as a major immunogen on the outer membrane of *Schistosoma mansoni* (Goudot-Crozel et al., 1989). A number of alternative functions have been described for GAPDH, including protein kinase activity (Kawamoto and Caswell, 1986), bundling of microtubules (Huitorel and Pantaloni, 1985), uracil DNA glycosylase activity (Meyer-Siegler et al., 1991), and binding of fibronectin, lysozyme, and cytoskeletal proteins (Pancholi and Fischetti, 1992).

These findings emphasise the need to provide more information on the possible roles and functions of these released, or surface-associated enzymes. What have been identified as laminin-binding protein (LBP), adhesins, surface cysteine proteinases, and according to our results, heme- and hemoglobin-binding proteins in *T. vaginalis*, might be only two or three chemical substances with different functional sites. Depending on what signal is received from the host environment, trichomonads might display one functional site within a surface molecule that might be concealed in the absence of its appropriate signal (López et al., 2000). The fact that expression of one of the *ap51* genes (*ap51-2*) is not regulated by iron, strengthen the idea that two isoforms of the same gene exist, one of which may be constitutively expressed while the second is environmentally regulated under the influence of iron (Alderete et al., 1998). Therefore, iron may be a good candidate to act as a cofactor to trigger the switching mechanism between the distinct functions of these proteins,

modulating their compartmentalization outside the hydrogenosomes (Moreno-Brito et al., 2005).

It appears that *T. vaginalis*, through developing multifunctional proteins, can take advantage of its limited genome in order to survive, and successfully parasitize in the constantly changing and nutrient-limiting environment of the vagina.

4.3. Conclusions

In this study, it was shown that *T. vaginalis* isolates were able to lyse sheep red blood cells regardless of virulence, and culture duration.

Two hemoglobin-binding proteins on the surface of *T. vaginalis* were identified, one of which has a higher affinity for heme moiety of hemoglobin, and therefore can be considered as a heme-binding protein for this parasitic protozoan. Amino acid sequence analysis of the 48- and 63-kDa proteins revealed identity to subunits of two *T. vaginalis* adhesins AP51 and AP65, respectively.

Functioning as heme- and hemoglobin-binding proteins, these two adhesive molecules likely play an important role in acquiring heme-iron during menstruation, when concentration of lactoferrin is at the lowest level. These two adhesins can be responsible for a portion of the binding and transporting of heme-iron into the cytoplasm of *T. vaginalis*, resulting in restoration of internal iron pools, which subsequently increases growth and multiplication of the parasite. This hypothesis may partially explain the reason that trichomoniasis symptoms exacerbate during or shortly after menstruation. However, it is still unclear how trichomonads are able to transport heme-iron through their membrane. Also, it has not been shown yet whether trichomonads are able to degrade the heme ring. Future studies are needed to answer these questions.

The heme- and hemoglobin-binding proteins along with three of *T. vaginalis* adhesins have identity with hydrogenosomal enzymes. This result reaffirms previous findings that *T. vaginalis* possess multifunctional proteins that are able to change their function upon changing their cellular localization. Functional diversity among trichomonal proteins, which may be regulated by iron, has a key role in enabling this parasite with a limited genome to withstand the complex and hostile environment of the human urogenital tract. Therefore, understanding the range of functions that these metabolic proteins can display may provide useful information leading to a better understanding of the *T. vaginalis* pathogenetic mechanisms.

LIST OF REFERENCES

- Addis, M.F., Rappelli, P., Cappuccinelli, P., and Fiori, P.L. (1997). Extracellular release by *Trichomonas vaginalis* of a NADP⁺ dependent malic enzyme involved in pathogenicity. *Microb. Pathog.* 23, 55-61.
- Addis, M.F., Rappelli, P., Delogu, G., Carta, F., Cappuccinelli, P., and Fiori, P.L. (1998). Cloning and molecular characterization of a cDNA clone coding for *Trichomonas vaginalis* alpha-actinin and intracellular localization of the protein. *Infect. Immun.* 66, 4924-4931.
- Addis, M.F., Rappelli, P., and Fiori, P.L. (2000). Host and tissue specificity of *Trichomonas vaginalis* is not mediated by its known adhesion proteins. *Infect. Immun.* 68, 4358-4360.
- Alderete, J.F. (1999). Iron modulates phenotypic variation and phosphorylation of P270 in double-stranded RNA virus-infected *Trichomonas vaginalis*. *Infect. Immun.* 67, 4298-4302.
- Alderete, J.F., and Kasmala, L. (1986). Monoclonal antibody to a major glycoprotein immunogen mediates differential complement-independent lysis of *Trichomonas vaginalis*. *Infect. Immun.* 53, 697-699.
- Alderete, J.F., Garza, G.E. (1988). Identification and properties of *Trichomonas vaginalis* proteins involved in cytoadherence. *Infect. Immun.* 56, 28-33.
- Alderete, J.F., Deměs, P., Gombošová, A., Valent, M., Yánoška, A., Fabušová, H., Kasmala, L., Garza, G.E., and Metcalfe, E.C. (1987). Phenotypes and protein-epitope phenotypic variation among fresh isolates of *Trichomonas vaginalis*. *Infect. Immun.* 55, 1037-1041.
- Alderete, J.F., Arroyo R., Dailey D.C., Engbring J., Khoshnan M.A., Lehker M.W., McKay J. (1992). Molecular analysis of *Trichomonas vaginalis* surface protein repertoires. *Mol. Cell. Biol. Hum. Dis. Ser. 1*:173-202.
- Alderete, J.F., Lehker, M.W., and Arroyo, R. (1995a). The mechanisms and molecules involved in cytoadherence and pathogenesis of *Trichomonas vaginalis*. *Parasitol. Today.* 11, 70-74.
- Alderete, J.F., Provenzano, D. and Lehkher, M.W. (1995b). Iron mediates *Trichomonas vaginalis* resistance to complement lysis. *Microb. Pathog.* 19, 93-103.
- Alderete, J.F., Engbring, J., Lauriano, C.M., and O'Brien J.L. (1998). Only two of the *Trichomonas vaginalis* triplet AP51 adhesins are regulated by iron. *Microb. Pathog.* 24, 1-16.
- Alderete, J.F., Millsap, K.W., Lehker, M.W., and Benchimol, M. (2001). Enzymes on microbial pathogens and *Trichomonas vaginalis*: molecular mimicry and functional diversity. *Cell. Micro.* 3, 359-370.
- Alderete, J.F., Nguyen, J., Mundodi, V. and Lehkher, M.W. (2004). Heme-iron increases levels of AP65-mediated adherence by *Trichomonas vaginalis*. *Microb. Pathog.* 36, 263-271.

Alvarez-Sánchez, M.E., Avila-González, L., Becerril-García, C. Fattel-Facenda, L.V., Ortega-López, J., and Arroyo. R. (2000). A novel cysteine proteinase (CP65) of *Trichomonas vaginalis* involved in cytotoxicity. *Microb. Pathog.* 28, 193-202.

Archambault, M., Labrie, J., Rioux, C.R., Dumas, F., Thibault, P., Elkins, C., and Jacques M. (2003). Identification and preliminary characterization of a 75-kDa hemin- and hemoglobin-binding outer membrane protein of *Actinobacillus pleuropneumoniae* serotype 1. *Can. J. Vet. Res.* 67, 271-277.

Arroyo, R., and Alderete, J.F. (1989). *Trichomonas vaginalis* surface proteinase activity is necessary for parasite adherence to epithelial cells. *Infect. Immun.* 57, 2991-2997.

Arroyo, R., Engbring, J., and Alderete, J.F. (1992). Molecular basis of host epithelial cell recognition by *Trichomonas vaginalis*. *Mol. Microbiol.* 6, 853-862.

Arroyo, R., Gonzalez-Robles, A., Martinez-Palomo, A., and Alderete, J.F. (1993). Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesion synthesis follows cytoadherence. *Mol. Microbiol.* 7, 299-309.

Aslan, D.L., Gulbahce, H.E., Stelow, E.B., Setty, S., Brown, C.A., McGlennen R.C., and Pambuccian, S.E. (2005). The diagnosis of *Trichomonas vaginalis* in liquid-based Pap tests: correlation with PCR. *Diagn. Cytopathol.* 32, 341-344.

Benchimol, M., Monteiro, S.P., Chang, T.-H., and Alderete, J.F. (2002). Virus in *Trichomonas*-an ultrastructural study. *Parasitol. Int.* 51, 293-298.

Bhatt, R., Abraham M., Petrin, D. Garber, G.E. (1996). New concepts in the diagnosis and pathogenesis of *Trichomonas vaginalis*. *Can. J. Infect. Dis.* 7, 321-325.

Bloom, B.R. (1979). Games parasites play: how parasites evade immune surveillance. *Nature* 279, 21-26.

Brugerolle, G., Bricheux, G., and Coffe, G. (2000). Immunolocalization of two hydrogenosomal enzymes of *Trichomonas vaginalis*. *Parasitol. Res.* 86, 30-35.

Budilova, M., and Kulda, J. (1977). Further studies on the effect of ferric ammonium citrate on the virulence of *Tritrichomonas foetus* to mice. *J. Protozool.* 24, 49.

Camara, M., Boulnois, G.J., Andrew, P.W., and Mitchell, T.J. (1994). A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein. *Infect. Immun.* 62, 3688-3695.

Carr, P.L., Felsenstein, D., and Friedman, R.H., (1998). Evaluation and management of vaginitis. *J. Gen. Intern. Med.* 13, 335-346.

- Carvalho, S.E., Freitas, D., Murad, A.M., Franco, O.L., and Simões-Barbosa, A. (2005) *Trichomonas vaginalis*: Identification of a triacylglycerol acylhydrolase. *Exp. Parasitol.* *111*, 260-263.
- Chen, C., Sparling P.F., Lewis, L.A., Dyer D.W., and Elkins, C. (1996). Identification and purification of a hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*. *Infect. Immun.* *64*, 5008-5014.
- Corbeil, L.B. (1995). Use of an animal model of trichomoniasis as a basis for understanding this disease in women. *Clin. Infect. Dis.* *21*(Suppl. 2), S158-S161.
- Cudmore, S.L., Delgaty, K.L., Hayward-McClelland, S.F., Petrin, D.P., and Garber, G.E. (2004). Treatment of Infections caused by metronidazole-resistant *Trichomonas vaginalis*. *Clin. Microbiol. Rev.* *17*, 783-793.
- Dailey, D.C., Chang, T.-H., and Alderete, J.F. (1990). Characterization of *Trichomonas vaginalis* haemolysis. *Parasitology* *101*, 171-175.
- Daniels, G.M., and Amara, S.G. (1998). Selective labeling of neurotransmitter transporters at the cell surface. *Methods Enzymol.* *296*, 307-318.
- Dashper, S.G., Hendtlass, A., Slakeski, N., Jackson, C., Cross, K.J., Brownfield, L., Hamilton, R., Barr, I., and Reynolds, E.C. (2000). Characterization of a novel outer membrane hemin-binding protein of *Prophyromonas gingivalis*. *J. Bacteriol.* *182*, 6456-6462.
- de Andrade Rosa, I., Einicker-Lamas, M., Bernardo, R.R., Previatto, L.M., Mohana-Borges, R., Morgado-Díaz, J.A., and Benchimol, M. (2006). Cardiolipin in hydrogenosomes: evidence of symbiotic origin. *Eukaryot. Cell* *5*, 784-787.
- de Carli, G.A., Brasseur, P., Tasca, T., da Silva, A., Castilhos, D.S., and Wendroff, A. (1998). Hemolytic aspects of strains and clones of *Trichomonas vaginalis*. *Parasitol. Día.* *22*, 59-64.
- Draper, D., Parker, R., Patterson, E., Jones, W., Beutz, M., French, J., Borchardt, K., and McGregor, J. (1993). Detection of *Trichomonas vaginalis* in pregnant women with the InPouch TV culture system. *J. Clin. Microbiol.* *31*, 1016-1018.
- Draper, D., Donohoe, W., Mortimer, L., and Heine, R.P. (1998). Cysteine proteases of *Trichomonas vaginalis* degrade secretory leukocyte protease inhibitor. *J. Infect. Dis.* *178*, 815-819.
- Engbring, J.A., Alderete, J.F. (1998). Three genes encode distinct AP33 proteins involved in *Trichomonas vaginalis* cytoadherence. *Mol. Microbiol.* *28*, 305-313.

- Fiori, P.L., Rappelli, P., Rocchigiani, A.M., and Cappuccinelli, P. (1993). *Trichomonas vaginalis* haemolysis: evidence of functional pores formation on red cell membranes. FEMS Microbiol. Lett. 109, 13-18.
- Fiori, P.L., Rappelli, P., Addis, M.F., Sechi, A., and Cappuccinelli, P. (1996). *Trichomonas vaginalis* haemolysis: pH regulates a contact-independent mechanism based on pore-forming proteins. Microb. Pathog. 20, 109-118.
- Fiori, P.L., Rappelli, P., Addis, M.F., Mannu, F., and Cappuccinelli, P. (1997). Contact-dependent disruption of the host cell membrane skeleton induced by *Trichomonas vaginalis*. Infect. Immun. 65, 5142-5148.
- Fiori, P.L., Rappelli, P., and Addis, M.F. (1999). The flagellated parasite *Trichomonas vaginalis*: new insight into cytopathogenicity mechanisms. Microbes. Infect. 1, 149-156.
- Fouts, A.C., and Kraus, S.J. (1980). *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. J. Infect. Dis. 141, 137-143.
- Fouz, B., Mazoy, R., Lemos, M.L., del Olmo, M.J., and Amaro, C. (1996). Utilization of hemin and hemoglobin by *Vibrio vulnificus* biotype 2. Appl. Environ. Microbiol. 62, 2806-2810.
- Fouz, B., Mazoy, R., Vázquez, F., Lemos, M.L., and Amaro, C. (1997). Isolation of a hemin and hemoglobin binding outer membrane protein of *Vibrio vulnificus* biotype 2 (serogroup E). FEMS Microbiol. Lett. 156, 187-191.
- Furano, K., Luke, N.R., Howlett, A.J., and Campagnari A.A. (2005). Identification of a conserved *Moraxella catarrhalis* haemoglobin-utilization protein, MhuA. Microbiology 151, 1151-1138.
- Furtado, M.B., Benchimol, M. (1998). Observation of membrane fusion on the interaction of *Trichomonas vaginalis* with human vaginal epithelial cells. Parasitol. Res. 84, 213-220.
- Frangipane, M.E., Morton, D.J., Wooten, J.A., Pozsgay, J.M., and Stull, T.L. (1994). Binding of human hemoglobin by *Haemophilus influenza*. FEMS Microbiol. Lett. 118, 243-248.
- Garber, G.E., and Lemchuk-Favel, L.T. (1989). Characterization and purification of extracellular proteases of *Trichomonas vaginalis*. Can. J. Microbiol. 35, 903-909.
- Garber, G.E., and Lemchuk-Favel, L.T. (1990). Association of production of cell-detaching factor with the clinical presentation of *Trichomonas vaginalis*. J. Clin. Microbiol. 28, 2415-2417.
- Garber, G.E., Lemchuk-Favel, L.T., and Bowie, W.R. (1989). Isolation of a cell-detaching factor of *Trichomonas vaginalis*. J. Clin. Microbiol. 27, 1548-1553.

- Garber, G.E., Lemchuk-Favel, L.T., and Rousseau, G. (1991). Effect of β -estradiol on production of the cell-detaching factor of *Trichomonas vaginalis*. J. Clin. Microbiol. 29,1847-1849.
- Garcia, A.F., Chang, T.-H., Benchimol, M., Klumpp, D.J., Lehker, M.W., and Alderete, J.F. (2003). Iron and contact with host cells induce expression of adhesins on surface of *Trichomonas vaginalis*. Mol. Microbiol. 47, 1207-1224.
- Garcia, A.F., Benchimol, M., and Alderete, J.F. (2005). *Trichomonas vaginalis* polyamine metabolism is linked to host cell adherence and cytotoxicity. Infect. Immun. 73, 2602-2610.
- Gifford, A.H.T., Klippenstein, J.R., and Moore, M.M. (2002). Serum stimulates growth of and proteinase secretion by *Aspergillus fumigatus*. Infect. Immun. 70, 19-26.
- Gilbert, R.O, Elia, G., Beach, D.H., Klaessig, S., and Singh, B.N. (2000). Cytopathogenic effect of *Trichomonas vaginalis* on human vaginal epithelial cells cultured in vitro. Infect. Immun. 68, 4200-4206.
- Gillin, F.D., and Sher, A. (1981). Activation of the alternative complement pathway by *Trichomonas vaginalis*. Infect. Immun. 34, 268-273.
- Gorrell, T.E. (1985). Effect of culture medium iron content on the biochemical composition and metabolism of *Trichomonas vaginalis*. J. Bacteriol. 161, 1228-1230.
- Goudot-Crozel, V., Caillol, D., Djabali, M., and Dessein, A.J. (1989). The major parasite surface antigen associated with human resistance to schistosomiasis is a 37-kDa glyceraldehyde 3P-dehydrogenase. J. Exp. Med. 170, 2065–2080.
- Guan, S., Nagata, H., Kuboniwa, M. Ikawa, Y., Maeda, K., and Shizukuishi, S. (2002). Characterization of binding and utilization of hemoglobin by *Prevotella nigrescens*. Oral. Microbiol. Immunol. 17, 157-162.
- Guan, S., Nagata, H., Maeda, K., Kuboniwa, M., Minamion, N., and Shizukuishi, S. (2004). Purification and characterization of a hemoglobin-binding outer membrane protein of *Prevotella intermedia*. FEMS Microbiol. Lett. 235, 333-339.
- Heath, B. (1980). Variant mitosis in lower eukaryotes: indicators of the evolution of mitosis? Int. Rev. Cytol. 64, 6–81.
- Heine, P., and McGregor, J.A. (1993). *Trichomonas vaginalis*: a reemerging pathogen. Clin. Obstet. Gynecol. 36, 137-144.
- Hobbs, M.M., Kazembe, P., Reed, A.W., Miller, W.C., Nkata, E., Zimba, D., Daly, C.C., Chakraborty, H., Cohen, M.S., and Hoffman, I. (1999). *Trichomonas vaginalis* as a cause of urethritis in Malawian men. Sex. Transm. Dis. 26, 381-387.

Hogue, M.J. (1943). The effect of *Trichomonas vaginalis* on tissue culture cells. Am. J. Hyg. 37, 142-152.

Honigberg, B.M., and Brugerolle, G. (1990). Structure. In *Trichomonads parasitic in humans*. B.M. Honigberg (ed.), pp 5-35. Springer-Verlag, New York, N.Y.

Horner, D.S., Foster, P.G., and Embley, T.M. (2000). Iron hydrogenases and the evolution of anaerobic eukaryotes. Mol. Biol. Evol. 17, 1695-1709.

Huitorel, P., and Pantaloni, D. (1985). Bundling of microtubules by glyceraldehyde-3-phosphate dehydrogenase and its modulation by ATP. Eur. J. Biochem. 150, 265-269.

Jesus, J.B., Ferreira, M.A., Cuervo, P., Britto, C., Silva-Filho, F.C., and Meyer-Fernandes, J.R. (2006). Iron modulates ecto-phosphohydrolase activities in pathogenic trichomonads. Parasitol. Int. 55, 285-290.

Joe, A., Murray, C.S., and McBride, B.C. (1994). Nucleotide sequence of a *Porphyromonas gingivalis* gene encoding a surface-associated glutamate dehydrogenase and construction of a glutamate dehydrogenase-deficient isogenic mutant. Infect. Immun. 62, 1358-1368.

Juliano, C., Monaco, G., Bandiera, P., Tedde, G., and Cappuccinelli, P. (1987). Action of anticytoskeletal compounds on in vitro cytopathic effect, phagocytosis, and adhesiveness of *Trichomonas vaginalis*. Genitourin. Med. 63, 256-63.

Kawamoto, R.M., and Caswell, A.H. (1986). Autophosphorylation of glyceraldehyde-phosphate dehydrogenase and phosphorylation of protein from skeletal muscle microsomes. Biochemistry 11, 657-661.

Kim, Y.S., Song, H.O., Choi, I.H., Park, S.J., and Ryu, J.S. (2006). Hydrogenosomal activity of *Trichomonas vaginalis* cultivated under different iron conditions. Korean J. Parasitol. 44, 373-378.

Kreiger, J.N., Poisson, M.A., and Rein, M.F. (1983). Beta-hemolytic activity of *Trichomonas vaginalis* correlates with virulence. Infect. Immun. 41, 1291-1295.

Kreiger, J.N., Tam, M.R., Stevens, C.E., Nielsen, I.O., Hale, J., Kiviat, N.B., and Holmes, K.K. (1988). Diagnosis of trichomoniasis. Comparison of conventional wet-mount examination with cytologic studies, cultures, and monoclonal antibody staining of direct specimens. JAMA 259, 1223-1227.

Krieger, J.N., Wolner-Hanssen, P., Stevens, C., and Holmes, K. K. (1990). Characteristics of *Trichomonas vaginalis* isolates from women with and without colpitis macularis. J. Infect. Dis. 161, 307-311.

- Kucknoor, A.S., Mundodi, V., and Alderete, J.F. (2005a). Heterologous expression in *Tritrichomonas foetus* of functional *Trichomonas vaginalis* AP65 adhesin. *BMC Mol. Biol.* 6, 5.
- Kucknoor, A.S., Mundodi, V., and Alderete, J.F. (2005b). Adherence to human vaginal epithelial cells signals for increased expression of *Trichomonas vaginalis* genes. *Infect. Immun.* 73, 6472-6478.
- Kulda, J. (1999). Trichomonads, hydrogenosomes and drug resistance. *Int. J. Parasitol.* 29, 199-212.
- Lahti, C.J., Bradley, P.J., and Johnson, P.J. (1994). Molecular characterization of the alpha-subunit of *Trichomonas vaginalis* hydrogenosomal succinyl CoA synthetase. *Mol. Biochem. Parasitol.* 66, 309-18.
- Lee, B.C. (1992). Isolation of an outer membrane hemin-binding protein of *Haemophilus influenzae* type b. *Infect. Immun.* 60, 810-816.
- Lehker, M.W., and Alderete, J.F. (1992). Iron regulates growth of *Trichomonas vaginalis* and the expression of immunogenic trichomonad proteins. *Mol. Microbiol.* 6, 123-132.
- Lehker, M.W., and Sweeney, D. (1999). Trichomonad invasion of the mucous layer requires adhesins, mucinases, and motility. *Sex. Transm. Inf.* 75, 231-238.
- Lehker, M.W., and Alderete, J.F. (2000). Biology of trichomonosis. *Curr. Opin. Infect. Dis.* 13, 37-45.
- Lehker, M.W., Chang, T.-H., Dailey, D.C., and Alderete, J.F. (1990). Specific erythrocyte binding is an additional nutrient acquisition system for *Trichomonas vaginalis*. *J. Exp. Med.* 171, 2165-2170.
- Lehker, M.W., Arroyo, R., and Alderete, J.F. (1991). The regulation by iron of the synthesis of adhesins and cytoadherence levels in the protozoan *Trichomonas vaginalis*. *J. Exp. Med.* 174, 311-318.
- León-Sicairos, N., Reyes-López, M., Canizalez-Román, A., Bermúdez-Cruz, R.M., Serrano-Luna, J., Arroyo, R., and de la Garza, M. (2005). Human hololactoferrin: endocytosis and use as an iron source by the parasite *Entamoeba histolytica*. *Microbiology* 151, 3859-3871.
- Lockwood, B.C., North, M.J., Scott, K.I., Bremner, A.F., and Coombs, G.H. (1987). The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. *Mol. Biochem. Parasitol.* 24, 89-95.
- López, L.B., de Melo Braga, M.B., López, J.O., Arroyo, R., and Silva-Filho, F.C., (2000). Strategies by which some pathogenic trichomonads integrate diverse signals in the decision-making process. *An. Acad. Bras. Ci.* 72, 173-186.

- Lottenberg, R., Broder, C.C., Boyle, M.D.P., Kain, S.J., Schroeder, B.L., and Curtis, III, R. (1992). Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. *J. Bacteriol.* 174, 5204-5210.
- Lubick, K.J., and Burgess, D.E. (2004). Purification and analysis of a phospholipase A2-like lytic factor of *Trichomonas vaginalis*. *Infect. Immun.* 72, 1284-1290.
- Mabey, D. Ackers, J, Adu-Sarkodie, Y. (2006). *Trichomonas vaginalis* infection. *Sex. Transm. Infect.* 82 (suppl IV), iv26-iv27.
- Mahmoud, M.S. (2002). Effect of the iron chelator deferoxamine on *Trichomonas vaginalis* in vitro. *J. Egypt. Soc. Parasitol.* 32, 691-704.
- Markos , A., Miretsky, A., and Müller, M. (1993) A glyceraldehydes-3-phosphate dehydrogenase with eubacterial features in the amitochondriate eukaryote , *Trichomonas vaginalis*, *J. Mol. Evol.* 37, 631-643.
- Mazoy, R., Vázquez, F., and Lemos, M.L. (1996). Isolation of heme-binding proteins from *Vibrio anguillarum* using affinity chromatography. *FEMS Microbiol. Lett.* 141, 19-23.
- McGrory, T., and Garber, G.E. (1992). Mouse intravaginal infection with *Trichomonas vaginalis* and role of *Lactobacillus acidophilus* in sustaining infection. *Infect. Immun.* 60, 2375-2379.
- Meyer-Siegler, K., Mauro, D.J., Seal, G., Wurzer, J., DeRiel, J.K., and Sirover, M.M. (1991). A human nuclear uracil DNA glycosylase is the 37 kDa subunit of glyceraldehydes-3-phosphate dehydrogenase. *Proc. Natl. Acad. Sci. USA.* 88, 8460-8464.
- Min, D.Y., Leem, M.H., Ahn, M.H., and Ryu, J.S. (1994). Identification of surface antigen of *Trichomonas vaginalis*. *Korean J. Parasitol.* 32, 243-248.
- Min, D.Y., Ryu, J.S., Park, S.Y., Shin, M.H., and Cho, W.Y. (1997). Degradation of human immunoglobulins and cytotoxicity on HeLa cells by live *Trichomonas vaginalis*. *Korean J. Parasitol.* 35, 39-46.
- Min, D.Y., Hyun, K.H., Ryu, J.S., Ahn, M.H., and Cho, M.H. (1998). Degradation of human immunoglobulins and hemoglobin by a 60 kDa cysteine proteinase of *Trichomonas vaginalis*. *Korean J. Parasitol.* 36, 261-268.
- Moreno-Brito, V., Yáñez-Gómez C., Meza-Cervantez, P., Ávila-González, L., Rodríguez, M.A., Ortega-López, J., González-Robles, A., and Arroyo, R. (2005). A *Trichomonas vaginalis* 120 kDa protein with identity to hydrogenosome pyruvate:ferredoxin oxidoreductase is a surface adhesin induced by iron. *Cell. Microbiol.* 7, 245-258.
- Müller, M. (1993). The hydrogenosome. *J. Gen. Microbiol.* 139, 2879-2889.

- Müller, M. (1997). Evolutionary origins of trichomonad hydrogenosomes, *Parasitol. Today* 13, 166-167.
- Müller, M., and Lindmark, R.G. (1976). Uptake of metronidazole and its effect on viability in trichomonads and *Entamoeba invadens* under anaerobic and aerobic conditions. *Antimicrob. Agents Chemother.* 9, 696-700.
- Mundodi, V., Kucknoor, A.S., Klumpp, D.J., Chang, T.-H., and Alderete, J.F. (2004). Silencing the *ap65* gene reduces adherence to vaginal epithelial cells by *Trichomonas vaginalis*. *Mol. Microbiol.* 53, 1099-1108.
- Mundodi, V., Kucknoor, A.S., Chang, T.-H., and Alderete, J.F. (2006). A novel surface protein of *Trichomonas vaginalis* is regulated independently by low iron and contact with vaginal epithelial cell. *BMC Microbiol.* 6, 6.
- Musatovova, O., and Alderete, J.F. (1998). Molecular analysis of the gene encoding the immunodominant phenotypically varying P270 protein of *Trichomonas vaginalis*. *Microb. Pathog.* 24, 223-239.
- Neale, K.A., and Alderete, J.F. (1990). Analysis of the Proteinases of Representative *Trichomonas vaginalis* Isolates. *Infect. Immun.* 58, 157-162.
- Nelson, D.A. (2000). Mechanism of iron acquisition from human transferrin by *Trichomonas vaginalis*. Thesis (MSc). The university of Texas, El Paso.
- Nielsen, M.H. (1976). In vitro effect of metronidazole on the ultrastructure of *Trichomonas vaginalis* Donné. *Acta Pathol. Microbiol. Scand. Sect. B* 84, 93-100.
- O'Brien, J.L., Lauriano, C., and Alderete, J.F. (1996). Molecular characterization of a third malic enzyme-like AP65 adhesin gene of *Trichomonas vaginalis*. *Microb. Pathog.* 20, 335-349.
- Ong, S.J., Huang, S.C., Liu, H.W., and Tai, J.H. (2004). Involvement of multiple DNA elements in iron-inducible transcription of the *ap65-1* gene in the protozoan parasite *Trichomonas vaginalis*. *Mol. Microbiol.* 52, 1721-1730.
- Pancholi, V., and Fischetti, V.A. (1992). A major surface protein on group A streptococci is a glyceraldehydes-3-phosphate-dehydrogenase with multiple binding activity. *J. Exp. Med.* 176, 415-426.
- Patel, S.R., Wiese, W., Patel, S.C., Ohl, C., Byrd, J.C., and Estrada, C.A. (2000). Systematic review of diagnostic tests for vaginal trichomoniasis. *Infect. Dis. Obstet. Gynecol.* 8, 248-257.
- Paterson, B.A., Tabrizi, S.N., Garland, S.M., Fairley, C.K., and Bowden, F.J. (1998). The tampon test for trichomoniasis: a comparison between conventional methods and a

polymerase chain reaction for *Trichomonas vaginalis* in women. Sex. Transm. Infect. 74, 136-139.

Peterson, K.M., and Alderete, J.F. (1982). Host plasma proteins on the surface of pathogenic *Trichomonas vaginalis*. Infect. Immun. 37, 755-762.

Peterson, K.M., and Alderete, J.F. (1984). Iron uptake and increased intracellular enzyme activity follow host lactoferrin binding by *Trichomonas vaginalis* receptors. J. Exp. Med. 160, 398-410.

Petrin, D., Delgaty, K., Bhatt, R., Garber, G. (1998). Clinical and Microbiological Aspects of *Trichomonas vaginalis*. Clin. Microbiol. Rev. 11, 300-317.

Pindak, F.F., Gardner, W.A.Jr., and Mora de Pindak, M. (1986). Growth and cytopathogenicity of *Trichomonas vaginalis* in tissue cultures. J. Clin. Microbiol. 23, 672-678.

Pindak, F.F., Mora de Pindak, M., and Gardner, W.A.Jr., (1993). Contact-independent cytotoxicity of *Trichomonas vaginalis*. Genitourin. Med. 69, 35-40.

Potamianos, S., Mason, P.R., Read, J.S., and Chikunguwo, S. (1992). Lysis of erythrocytes by *Trichomonas vaginalis*. Biosci Rep. 12, 387-395.

Provenzano, D., and Alderete, J.F. (1995). Analysis of human immunoglobulin-degrading cysteine proteinases of *Trichomonas vaginalis*. Infect. Immun. 63, 3388-3395.

Radonjic, I.V, Dzamic, A.M., Mitrovic, S.M., Arsic Arsenijevic, V.S., Popadic, D.M., and Kranjic Zec, I.F. (2006). Diagnosis of *Trichomonas vaginalis* infection: The sensitivities and specificities of microscopy, culture and PCR assay. Eur. J. Obstet. Gynecol. Reprod. Biol. 126, 116-120.

Rappelli, P., Rocchigiani, A.M., Erre, G., Colombo, M.M., Cappuccinelli, P., and Fiori, P. (1995). Sequence of cDNA coding for a 65 kDa adhesive protein for the specific detection of *Trichomonas vaginalis* by PCR. FEMS Microbiol. Lett. 129, 21-26.

Rendón-Maldonado, J.G, Espinosa-Cantellano, M., González-Robles, A., and Martinez-Palomo, A. (1998). *Trichomonas vaginalis*: in vitro phagocytosis of lactobacilli, vaginal epithelial cells, leukocytes, and erythrocytes. Exp. Parasitol. 89, 241-250.

Rendón-Maldonado, J., Espinosa-Cantellano, M., Soler, C., Torres, J.V., and Martinez-Palomo, A. (2003). *Trichomonas vaginalis*: in vitro attachment and internalization of HIV-1 and HIV-1-infected lymphocytes. J. Eukaryot. Microbiol. 50, 43-48.

Rosset, I., Tasca, T., Tessele, P.M., and De Carli, G.A. (2002). Scanning electron microscopy in the investigation of the in vitro hemolytic activity of *Trichomonas vaginalis*. Parasitol. Res. 88, 356-359.

- Ryu, J.S., and Min, D.Y. (2006). *Trichomonas vaginalis* and trichomoniasis in the Republic of Korea. *Korean J. Parasitol.* *44*, 101-116.
- Ryu, J.S., Choi, H.K., Min, D.Y., Ha, S.E., and Ahn, M.H. (2001). Effect of iron on the virulence of *Trichomonas vaginalis*. *J. Parasitol.* *87*, 457-460.
- Sajid, M., and McKerrow, J.H. (2002). Cysteine proteases of parasitic organisms. *Mol. Biochem. Parasitol.* *120*, 1-21.
- Schwebke, J.R., and Burgess, D. (2004). Trichomoniasis. *Clin. Microbiol. Rev.* *17*, 794-803.
- Schwebke, J.R., and Lawing, L.F. (2002). Improved detection by DNA amplification of *Trichomonas vaginalis* in males. *J. Clin. Microbiol.* *40*, 3681-3683.
- Scott, D.A., North, M.J., and Coombs, G.H. (1995). The pathway of secretion of proteinases in *Trichomonas vaginalis*. *Int. J. Parasitol.* *25*, 657-666.
- Seña, A.C., Miller, W.C., Hobbs, M.M., Schwebke, J.R., Leone, P.A., Swygard, H., Atashili, J., and Cohen, M.S. (2007). *Trichomonas vaginalis* infection in male sexual partners: implications for diagnosis, treatment, and prevention. *Clin. Infect. Dis.* *44*, 13-22.
- Sengupta, S., Tripathi, J., Tandon, R., Raje, M., Roy, R.P., Basu, S.K., and Mukhopadhyay, A. (1999). Hemoglobin endocytosis in *Leishmania* is mediated through a 46-kDa protein located in the flagellar pocket. *J. Biol. Chem.* *274*, 2758-2765.
- Serrano-Luna, J.J., Negrete, E., Reyes, M., and de la Garza, M. (1998). *Entamoeba histolytica* HM1:IMSS: hemoglobin-degrading neutral cysteine proteases. *Exp. Parasitol.* *89*, 71-77.
- Sommer, U., Costello, C.E., Hayes, G.R., Beach, D.H., Gilbert, R.O., Lucas, J.J., and Singh, B.N. (2005). Identification of *Trichomonas vaginalis* cysteine proteases that induce apoptosis in human vaginal epithelial cells. *J. Biol. Chem.* *280*, 23853-23860.
- Soper, D. (2004). Trichomoniasis: under control or undercontrolled? *Am. J. Obstet. Gynecol.* *190*, 281-290.
- Sorvillo, F., Smith, L., Kerndt, P., and Ash, L. (2001). *Trichomonas vaginalis*, HIV, and African-Americans. *Emerg. Infect. Dis.* *7*, 927-932.
- Swygard, H., Seña, A.C., Hobbs, M.M., and Cohen, M.S. (2004). Trichomoniasis: clinical manifestations, diagnosis and management. *Sex. Transm. Infect.* *80*, 91-95.
- Tachezy, J., Kulda, J., Bahníková, I., Suchan, P., Rázga, J., and Schrével, J. (1996). *Trichomonas foetus*: Iron acquisition from Lactoferrin and Transferrin. *Exp. Parasitol.* *83*, 216-228.

- Tasca, T., and De Carli, G.A. (1999). Hemolytic activity of fresh isolates and clones of *Trichomonas gallinae*. *Parasitol. al Dia*. 23, 69-73.
- Tsai, C.D., Liu, H.W., and Tai, J.H. (2002). Characterization of an iron-responsive promoter in the protozoan pathogen *Trichomonas vaginalis*. *J. Biol. Chem.* 277, 5153-5162.
- Upcroft, P., and Upcroft, J.A. (2001). Drug targets and mechanisms of resistance in the anaerobic protozoa. *Clin. Microbiol. Rev.* 14, 150-164.
- Vargas-Villarreal, J., Mata-Cárdenas, B.D., González-Salazar, F., Lozano-Garza, H.G., Cortes-Gutierrez, E.I., Palacios-Corona, R., Martínez-Rodríguez, H.G., Ramírez-Bon, E., and Said-Fernández, S. (2003). *Trichomonas vaginalis*: identification of a phospholipase A-dependent hemolytic activity in a vesicular subcellular fraction. *J. Parasitol.* 89, 105-112.
- Vargas-Villarreal, J., Mata-Cárdenas, B.D., Palacios-Corona, R., González-Salazar, F., Cortes-Gutierrez, E.I., Martínez-Rodríguez, H.G., and Said-Fernández S. (2005). *Trichomonas vaginalis*: identification of soluble and membrane-associated phospholipase A1 and A2 activities with direct and indirect hemolytic effects. *J. Parasitol.* 91, 5-11.
- Wang, A.L., and Wang, C.C. (1985). A linear double-stranded RNA in *Trichomonas vaginalis*. *J. Biol. Chem.* 260, 3697-3702.
- Wang, A., Wang, C., and Alderete, J.F. (1987). *Trichomonas vaginalis* phenotypic variation occurs only among trichomonads with the double-stranded RNA virus. *J. Exp. Med.* 166, 142-150.
- World Health Organization. (2001). Global prevalence and incidence of selected curable sexually transmitted infections. World Health Organization, Geneva, Switzerland.
- Wilson, M.E., and Britigan, B.E. (1998). Iron acquisition by parasitic protozoa. *Parasitol. Today* 14, 348-353.
- Zuo, Y., Riley, D.E., and Krieger, J.N. (1999). Flagellar duplication and migration during the *Trichomonas vaginalis* cell cycle. *J. Parasitol.* 85, 203-207.