PATHOGENIC EFFECT OF *TRICHOMONAS VAGINALIS* ON VARIOUS CELL LINES *IN VITRO*

by

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Submitted in partial fulfilment of

the requirements for the degree of

Master of Medical Science

in the

Department of Medical Microbiology

University of KwaZulu-Natal

Durban

2010

DECLARATION

The content of this dissertation represents the student's original work which has not been submitted in any form to any other University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this thesis was carried out at the Medical Microbiology research Laboratories, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, under the supervision of Professor A. W. Sturm.

______________________________ ______________________________

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PUBLICATIONS AND PRESENTATIONS EMANATING FROM THIS DISSERTATION

1. Presented at the Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) 2007 conference held in Cape Town.

2. Poster presentation at the Astra-Zeneca Research symposium, University of KwaZulu-Natal, held at the University of Durban Westville in 2007.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude and thanks to the following individuals:

My supervisor Prof A. W. Sturm, Department of Medical Microbiology, University of KwaZulu-Natal, for his guidance and constructive criticism during preparation of this thesis.

The laboratory staff of the Department of Medical Microbiology, University of KwaZulu-Natal, for their assistance and consistent encouragement for the duration of my laboratory work.

Mrs Tonya M. Esterhuizen, Biostatician/senior lecturer, Programme of Biostatics, Research Ethics and Medical Law, College of Health Science, University of KwaZulu-Natal, for her assistance in analysing data.

The National Research Foundation for partially providing financial assistance for my studies.

Finally, a special thanks to my husband and family especially my parents Hemmy and Shirley Bhojraj for their support, patience, and words of encouragement throughout the last four years.

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KEY TO ABBREVIATIONS

BV	Bacterial vaginosis
CPs	Cysteine proteinases
DMSO	Dimethylsuphoxide
FCS	Foetal calf serum
FN	Fibronectin
HCl	Hydrochloric acid
hrs	Hours
LDH	Lactate dehydrogenase
kDa	Kilodalton
µg	Microgram
µl	Microlitre
µm	Micrometer
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
MAb	Monoclonal antibody
MOI	Multiplicity of Infection
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PID	Pelvic Inflammatory Disease
PBS	Phosphate buffered saline
rpm	Revolutions per minute
SA	South Africa

STD	Sexually transmitted disease
STIs	Sexually transmitted infections
TLCK	N-alpha-p-tosyl-L-lysine chloromethyl ketone
NaOH	Sodium Hydroxide
EDTA	Ethylene-diamine-tetra-acetic acid
i.e	That is
ValRS	Valyl-tRNA-synthetase
VECs	Vaginal epithelial cells
via.	By means of
viz.	Namely

ABSTRACT

Trichomoniasis has been linked to pelvic inflammatory disease, cervical cancer, increased HIV transmission, infertility as well as co-infections with other STIs. In 2002, an association was found with *Trichomonas vaginalis* and PID in HIV positive women. Therefore, the question arose whether *T. vaginalis* is able to invade the upper genital tract of HIV infected women. A prerequisite for invasion of the upper genital tract is the capability of the organism to adhere to the cells of the organs involved. This study therefore investigated the interaction between *T. vaginalis* and vaginal, cervical and endometrial cells.

In comparing adhesion and cytotoxicity of *T. vaginalis* to cells of the upper and lower genital tract at different pH, immortalized vaginal (VK2), cervical (ME 180) and endometrial (KLE) cells were exposed to a standardized inoculum of trichomonads at pH 4.5 to 7.0. Adhesion was measured microscopically after acridine orange staining and cytotoxicity was established by measuring LDH release using a commercial kit.

Adhesion of the ME-180 and VK2 cell lines was found to be pH dependent. However, the KLE cell line was not. As the pH increased, adherence to the vaginal and cervical cells decreased. Adhesion to endometrial cells was minimal at neutral pH but marked adhesion was found at lower pH. For the vaginal cell line, cytotoxicity was minimal at pH 4.5 but substantial (30 to 60%) at higher pH. In contrast, cytotoxicity on cervical and endometrial cells was highest at lower pH.

The pronounced toxicity of vaginal epithelial cells at pH 5 and pH 5.5 is in keeping with the pH range found in patients with vaginitis. The observations on the cervical epithelium suggest toxic effect on the ecto-cervical epithelium immediate after acquisition of the infection. Adhesion of trichomonads to the endometrial cell line suggests that *T. vaginalis* is capable of colonization of the upper genital tract. At pH values applicable to the *in vivo* situation, toxicity was very low.

CHAPTER 1

INTRODUCTION

Sexually transmitted infections (STIs) are transmitted almost entirely via sexual contact with an infected person (Krieger & Alderete, 1999). The most common infectious agents causing STIs are *Neisseria gonorrhoeae*, the oculogenital biovars of *Chlamydia trachomatis*, Herpes simplex virus 2 and *Trichomonas vaginalis.*

Trichomonas vaginalis is the only protozoal cause of sexually transmitted infections (Petrin *et al.,* 1998). Trichomoniasis, the disease caused by *T. vaginalis* is characterized by an abnormal vaginal discharge. The inflammation of the vaginal epithelium results in redness, swelling and leukocyte infiltration (Zariffard *et al.*, 2004). More than 170 million people worldwide each year are infected by this STI (Ryu & Min, 2006). Trichomoniasis is one of the most commonly reported STIs in females in KwaZulu-Natal, South Africa (Moodley & Sturm, 2004). The prevalence has been reported as 28% among STD clinic attendees and 20% among antenatal clinic attendees (Moodley & Sturm, 2004).

An association between *T. vaginalis* infection and reproductive tract complications such as premature rupture of membranes has been reported (Cotch *et al.*, 1997). Trichomoniasis is linked to various inflammatory diseases such as prostatitis, pelvic inflammatory disease (PID) as well as to increased HIV transmission (Draper *et al.*, 1998; Petrin *et al.*, 1998; Moodley *et al.*, 2002; Skerk *et al.*, 2002). It is also associated with infertility in men and women as well as co-infections with other STIs (Petrin *et al.*, 1998).

Pelvic inflammatory disease is caused by micro-organisms such as *Neisseria gonorrhoeae, Chlamydia trachomatis* and the bacterial vaginosis associated organisms (Moodley *et al.*, 2002). In symptomatic women, the spread of infection from the cervix to the endometrium,

uterine tubes and concomitant structures results in abdominal pain accompanied by painful urination, vaginal discharge and fever (CDC, 1995; CDC, 2006). The association of *T. vaginalis* and pelvic inflammatory disease in HIV positive women, suggests that co-infection with HIV may alter the host-microbe relationship resulting in an increased risk of PID (Moodley *et al.*, 2002). The authors hypothesize that either HIV infection allows *T. vaginalis* to migrate into the internal upper genitalia or that vaginal trichomoniasis facilitates ascending bacterial infections in HIV infected women. One of the factors that might facilitate migration up the genital tract is the possibility of *T. vaginalis* to attach to the cells of the upper genital tract. This study addresses the question whether *T. vaginalis* is able to adhere to the cells of the upper genital tract and whether the effect of interaction with these cells differs from those of the lower genital tract.

CHAPTER 2

LITERATURE REVIEW

2.1 History and morphology of *Trichomonas vaginalis*

2.1.1 History

Little is known about the discovery and history of *Trichomonas vaginalis*. In 1836, Alfred Francois Donné microscopically observed both the appearance and size of the protozoan which he referred to as '*Trichomonas*' based on its similarity with Tricodes and Monas (Thorburn, 1974). Later, Ehrenberg, a professor of Protozoology pointed out that the protozoan's human habitat is vaginal and therefore specifically named the protozoan *Trichomonas vaginalis* (Thorburn, 1974).

Trichomonas vaginalis was initially considered to be non-pathogenic because most of the infected patients were asymptomatic. In 1942, Hesseltine found that 13% of female patients inoculated with *T. vaginalis* culture had developed trichomoniaisis (Hesseltine *et al.*, 1942). In 1953, Lanceley & McEntegart inoculated five male volunteers with *T. vaginalis* culture, three of which developed urethritis (Lanceley & McEntegart, 1953).

2.1.2 Morphology

Trichomonas vaginalis is a prokaryotic organism with a eukaryotic nucleus. It belongs to the Sub-Kingdom Protozoa and Sub-Phylum Mastigophora, which means "Flagella bearers". The family of Trichomonadidae consists of seven genera: *Trichomonas, Pentatrichomonas, Tetratrichomonas, Pentatrichomonoides, Trichomitopsis, Pseudotrypanosoma* and *Cochlosoma* (Cepicka *et al.*, 2006). *Trichomonas vaginalis* is the largest of three species of the genus *Trichomonas* inhabiting man. The other two are *Trichomonas hominis* and *Trichomonas tenax*. *Trichomonas vaginalis* is characterized by having trophozoites or feeding stages only (Petrin *et al.*, 1998). This means that no cystic, environmental resistant form

exists. This explains the need for transmission through direct contact between mucosal surfaces.

The organism as shown in Figure 2.1 is microscopic in size (approximately 10µm long and 7µm wide) and complex in structure, physiology and behaviour. The oval trophozoite has a short undulating membrane, four flagella (filamentous outgrowths of the ectoplasm) and a clearly visible barb-like axostyle (Alderete *et al*., 1995c). The axostyle may be used for surface attachment to vaginal epithelial cells (VECs) causing tissue damage (Petrin *et al.,* 1998). *Trichomonas vaginalis* also has a basic cytostome or gullet (mouth aperture) which helps to maintain the intraluminal position. *In vitro*, trichomonal cells appear ovoid or pear-shaped whereas in the presence of VECs, they display an amoeboid appearance (Arroyo *et al*., 1993). In culture, the dividing growth phase contains several oversized round forms either without flagella or with flagella equipped with a dividing nucleus or multiple nuclei.
Trichomonas vaginalis is highly sensitive to both atmospheric oxygen and the lack of moisture and will therefore not survive for more than a few hours outside of the body.

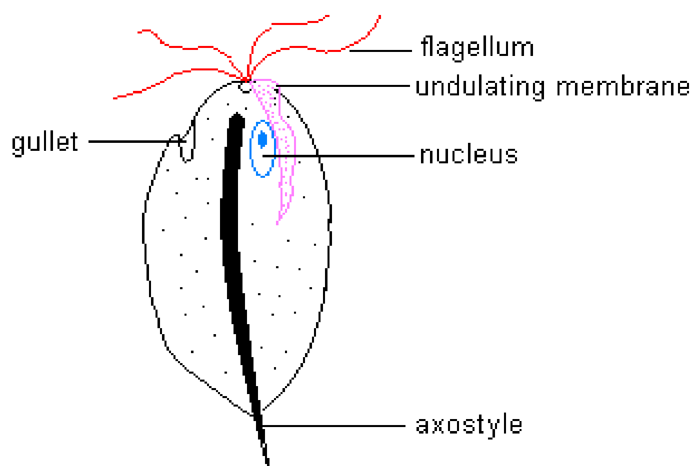


Figure 2.1 An illustration of *Trichomonas vaginalis*

2.2 Reproduction and life cycle

Trichomonas vaginalis multiplies by means of longitudinal binary fission. While the nuclear divisions in other protozoans are either mitotic or meiotic, trichomonads are an exception. They reproduce by a special type of closed mitosis called cryptopleuromitosis, a process similar to that of prokaryotic cells. Figure 2.2 illustrates the process of cryptopleuromitosis which involves the development of spindle microtubules from two attractophores flanking the nucleus. These spindle microtubules extend into the nucleus and attach to the kinetochores of the chromosomes. These spindle microtubules also elongates between the two attractophores ultimately separating the two daughter cells. Each daughter cell reconstructs by producing any missing organelles (Petrin *et al.*, 1998; Noël *et al.,* 2003).

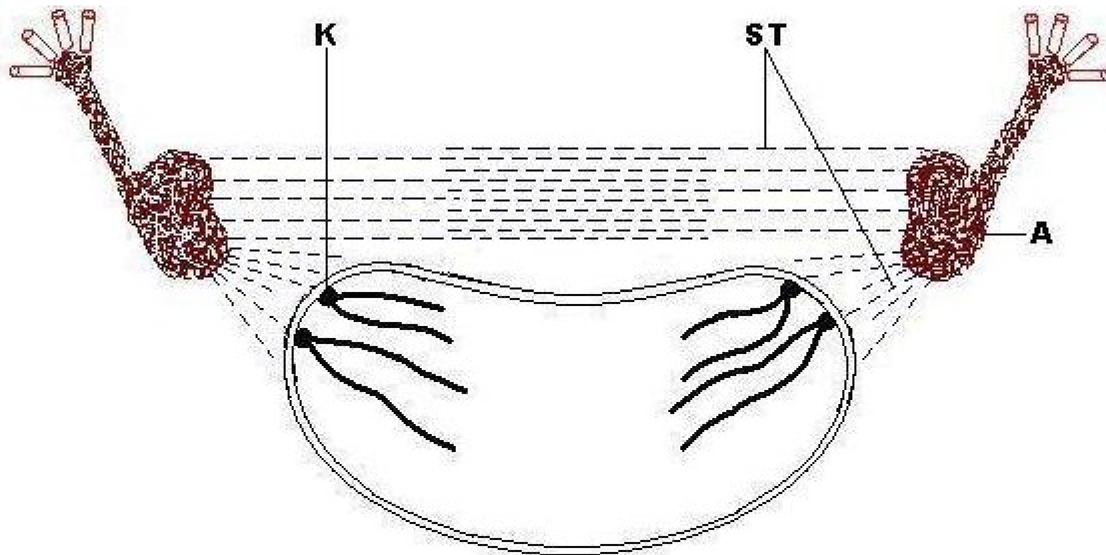


Figure 2.2 The process of cryptopleuromitosis. The spindle tubules (ST) stretch between two attractophores (A) and the kinetochores (K) of the surface of the nucleus.

2.3 Cell function

Trichomonas vaginalis lacks important eukaryotic organelles, such as mitochondria, peroxisomes and enzymes needed to conduct oxidative phosphorylation. Nutrients are obtained by both phagocytosis and transport through the cell membrane. *Trichomonas vaginalis* produces adenosine triphosphate (ATP), by utilizing enzymes typically found in anaerobic bacteria. They do so by means of a unique, double membrane-bound organelle called the hydrogenosome. These anaerobic mitochondria equivalents divide via fission and produce ATP via substrate phosphorylation (Palmer, 1997). However, they differ from real mitochondria in that they do not contain genetic material and lack cytochromes (Palmer, 1997).

The controversy surrounding mitochondrial symbiosis and divergence of trichomonads from the main eukaryotic tree was resolved by a study showing the presence of VaIRS proteins and mitochondrial heat shock proteins in *T. vaginalis*. This study suggests that mitochondrial symbiosis had occurred first (Hashimoto *et al.*, 1998; Germot & Philippe, 1999).

2.4 Epidemiology

Trichomoniasis is a sexually transmissible infection that is capable of causing substantial morbidity (Valadkhani *et al.*, 2003). *Trichomonas vaginalis* is the only pathogenic species of the genus *Trichomonas* and has a worldwide distribution. The disease affects individuals in all racial groups and depending on the type of sub-population studied prevalence rates vary between 0 and 65% (Lossick, 1990a). In men, the organism can be found in the urethra, periurethral glands, prostate, epididymis, and in semen (Krieger, 1995).

In 2001, WHO estimated that 174 million cases occur worldwide annually, making *T. vaginalis* infection the most common non-viral STI (WHO, 2001). In the US, trichomoniasis affects two to three million women annually. Reports from China indicate that it accounts for more than 30% of gynaecologic inflammatory diseases causing female sterility (Chen *et al.*, 2004). A Korean study done on patients complaining of vaginal symptoms found that 10.4% of these patients were infected by *T. vaginalis* (Ryu & Min, 2006).

The prevalence of trichomoniasis at STD clinics varies from eight to 31% (WHO, 2001). In KwaZulu-Natal, trichomoniasis is one of the most commonly reported STIs in females. A prevalence rate of 28% among STD clinic attendees was reported in 2004 (Moodley & Sturm, 2004).

It was found that the prevalence of trichomoniasis was lower in women attending antenatal clinics (Johnson *et al.*, 2005). Studies done, amongst pregnant women in South Africa show prevalence rates between 15 and 41.4% (Sturm *et al.*, 1998; Moodley & Sturm, 2004; Frohlich *et al.*, 2007).

These figures underestimate the real prevalence, since up to one half of all infections are asymptomatic and never diagnosed. Asymptomatic carriers can unknowingly transmit *T. vaginalis* via unprotected sexual contact. Therefore, asymptomatic infections are epidemiologically important. Sexual intercourse is the most common route of transmission. *Trichomonas vaginalis* can survive for several hours outside the host; however, non-venereal transmission is extremely rare (Heine & McGregor, 1993).

2.5 Clinical manifestation, diagnosis and treatment

2.5.1 Clinical manifestation

Trichomonas vaginalis mainly infects the squamous epithelium of the female genital tract. This organism has previously been found in the urethra, fallopian tubes and the pelvis (Upcroft & Upcroft, 2001). Clinically trichomoniasis varies from an asymptomatic carrier state to overt vaginitis. This however, depends on the severity of the disease (Wisdom & Dunlop, 1965). One third of asymptomatic women display clinical trichomoniasis within six months (Heine & McGregor, 1993).

Acute trichomoniasis is characterized by diffuse vulvitis and vaginitis with pruritis, dyspareunia and dysuria (Heine & McGregor, 1993). Small punctuate haemorrhagic spots often appear on the cervical mucosa, a situation termed the "strawberry cervix" (Wolner-Hanssen *et al.*, 1989; Rein, 1989). Symptoms include a profuse, mucopurulent, frothy, malodorous, yellow or greenish discharge (Wolner-Hanssen *et al.*, 1989; Petrin *et al.*, 1998). In this stage, the lactobacilli which form the normal vaginal flora have been replaced by anaerobic flora similar to that found in bacterial vaginosis. However, asymptomatic women have a normal vaginal pH with normal vaginal flora (Wisdom & Dunlop, 1965; Fouts & Kraus, 1980). This state of infection is termed the "carrier stage".

The clinical signs exacerbate with menstruation and often improve during pregnancy (Heine & McGregor, 1993). It is thought that menstrual blood causes a rise in pH creating an ideal environment for trichomonad reproduction (Goode *et al.*, 1994). Also, the elevated iron concentration in menstrual fluid aids in activating various pathogenic mechanisms, such as cysteine proteinases (Petrin *et al.*, 1998). The hormone estrogen, which levels are decreased

during menstruation, has been shown to inhibit a pathological protein known as cell detaching factor (Garber *et al.*, 1991).

This organism has been associated with reproductive tract complications, such as premature rupture of membranes, low birth weight, cervical cancer, infertility, as well as HIV transmission (Rein, 1989; Heine & McGregor, 1993; Draper *et al.*, 1998). Trichomoniasis is predominant in women, but men are considered as asymptomatic carriers of this disease. Symptomatic cases, such as urethral discharge, urethritis and prostatitis have been reported in 33% to 50% of male trichomonal infections (Krieger, 1995). Also noted is the link between *T. vaginalis* and various complications including pelvic inflammatory disease and infertility (Krieger, 1995).

2.5.2 Diagnosis

Clinical diagnosis is unreliable because symptoms and signs are often similar to those of other STDs (Rein & Holmes, 1983). For example, an elevated vaginal pH measured using Nitrazine paper is suggestive of both trichomoniasis and bacterial vaginosis (BV). The release of a fishy odour upon application of 10% potassium hydroxide (potassium hydroxide amine test) is also suggestive of both trichomoniasis and BV.

Trichomoniasis, is therefore diagnosed by means of laboratory tests like microscopic examination of the discharge, preferably using staining techniques, by culture or by polymerase chain reaction (PCR). Polymerase chain reaction based tests have a sensitivity and specificity in the order of 97% to 98% (Heine *et al.*, 1997; Lin *et al.*, 1997). Recently developed, was a diagnostic PCR which detects low concentrations of organisms by

incorporating primers based on repetitive sequences in the *T. vaginalis* genome (Ryu & Min, 2006). In addition, oligonucleotide probes, monoclonal antibody dot blot assays and many more have been developed to detect *T. vaginalis* (Gombosova & Valent, 1990; Briselden & Hillier, 1994; Muresu *et al.*, 1994).

The 'gold standard' broth culture is by far the most efficient, easy, valuable and economical tool for obtaining an accurate diagnosis (Lossick & Kent, 1991; Bhatt *et al.*, 1996; Carr *et al.*, 1998). However, culture needs an incubation period of two days. Therefore, culture is not suitable for patient management as treatment needs to be commenced immediately. Monoclonal antibodies (MAbs) have been used for rapid detection as well as identification of isolates of cultured *T. vaginalis* (Krieger *et al.*, 1985a). Tests, such as *Trichomonas* Direct Enzyme Immunoassay may be completed within one hour, permitting immediate diagnosis and treatment. However, the sensitivity of these tests is lower than that of culture or PCR.

2.5.3 Treatment

Metronidazole, a 5-nitroimidazole is currently used for the treatment of human urogenital trichomoniasis (Hrdy *et al.*, 2005). Even though other members of the nitroimidazole class are structurally related and differ in pharmacokinetic and therapeutic activities, they can also be used to treat this infection (Lau *et al.*, 1992; Mathisen & Finegold, 1992; Gillis & Wiseman, 1996). A single oral dose of 2g metronidazole effectively treats trichomoniasis (CDC, 2006). However, this single dose is associated with gastrointestinal side effects and has a sub-optimal effect on Bacterial vaginosis (BV).

Because there is no protective immunity, re-infections are common. In preventing re-infection and reducing transmission, it is recommended that both partners be concurrently treated (Swygard *et al.*, 2004; CDC, 2006).

There is emergence of metronidazole resistance *in vivo* and *in vitro*. Prevalence rates of 2.5% and 5% have been reported (Schwebke & Barrientes, 2006). However, the correlation between this and non-response to treatment (*in vivo* resistance) is not clear and needs further elucidation. Several different dose regimens have been proposed to eradicate metronidazole resistant trichomoniasis. It has been suggested, to increase the dose of the drug in the case of refractory infection. Some cases require prolonged treatment with such high doses that these need to be intravenously administered in order to reduce gastrointestinal side effects (Lossick, 1990b; Narcisi & Secor, 1996).

Metronidazole activation occurs within the hydrogenosome where it is reduced to cytotoxic anion radicals by pyruvate-ferredoxin oxidoreductase. The electrons required for activation are released from pyruvate and transferred to the nitro group of metronidazole by ferredoxin, a low-redox-potential carrier (Hrdy *et al.*, 2005). This reductive activation creates a concentration gradient, allowing more of the drug to enter the cell. These highly reactive radicals cause damage to cellular DNA resulting in death (Edwards, 1993). It has been hypothesized that poorly functional hydrogenosomal oxidases, in resistant strains, results in an increased oxygen concentration. Oxygen, an important competitor for available electrons, competes with the drug resulting in little reduction of the nitro group, oxidizing the nitro-free radicals to the original compound (Lewis *et al.*, 1997).

2.6 Host defence mechanisms

Although recurrent infections are common, the infection does not appear to confer immunity. However, immunity to *T. vaginalis* infection may be important in restricting the organism to the genito-urinary tract by stimulating non-specific responses (Ackers, 1990). These responses include complement activation via the alternative pathway as well as neutrophil phagocytosis (Shaio & Lin, 1995; Petrin *et al.*, 1998).

Infection caused in men is short-lived and this could be due to the presence of zinc in the prostatic fluid which has been shown to be cytotoxic to *T. vaginalis,* and therefore may be an inhibiting factor (Daly *et al.*, 1990). Also, the reducing environment of the vagina is sufficient to activate proteinases involved in pathogenesis. However, the male genital tract is oxidative, thereby inhibiting such mechanisms (Alderete & Provenzano, 1997).

The ecology of the vaginal tract during the reproductive years proves highly significant in determining the severity of infection. Infection in the pre-menarchal girl and post-menopausal women is generally mild and transient as compared to women of the reproductive age. This is due to possible pathogenic factors, such as the vaginal pH, iron concentrations in menstrual flow and estrogen fluctuations (Rein, 1989). Dependability of the organism to iron concentrations in the secretions is apparent. When iron supply is low, it migrates deeper into the female genital tract in search of richer iron supply resulting in infection (Rein, 1989). It is amazing how this organism survives dramatic environmental changes, such as the influx of host macromolecules, erythrocytes, serum constituents and pH changes. Research focusing on the initial events of infection is essential in understanding how the organism establishes infection.

2.7 Pathogenesis

Trichomonas vaginalis elaborates several mechanisms that contribute to pathogenesis during infection (Zariffard *et al.*, 2004). Being the most intensely studied trichomonad, these pathogenic mechanisms have not been clearly elucidated. Mechanisms utilized by this parasite include: interaction with the vaginal flora, immune evasion, recognition and binding to mucin, cytoadherence mediated by adhesins, haemolysis and the excretion of soluble factors, degrading all immunoglobulin subclasses and complement (Petrin *et al.*, 1998; Lehker & Sweeney, 1999; Alderete *et al.*, 2002). The surface of the organism is a vital component of the complex host-parasite interaction. The fact that *T. vaginalis* is capable of long term survival in the adverse vaginal milieu demonstrates the highly evolved nature of this organism (Alderete *et al.*, 2002). Focusing on the initial stages of infection provides a clearer understanding of the pathogenesis of *T. vaginalis.* This in turn, could aid in developing new intervention strategies, better treatment, and better disease control.

2.7.1 Interaction with vaginal flora

The normal vaginal pH is very acidic (4.5 to 5.0) but *T. vaginalis* can survive in this milieu. The increase in pH due to infection results in a decrease or complete loss of vaginal lactobacilli and an increase in anaerobic bacteria. This inverse relationship between *T. vaginalis* and vaginal lactobacilli is not completely understood. *Trichomonas vaginalis* is capable of phagocytising bacteria, including lactobacilli (Rendon-Maldonado *et al.*, 1998). Furthermore, soluble factors secreted by *T. vaginalis* such as proteinases may destroy them.

2.7.2 Immune evasion

An important aspect of pathogenesis is the ability to evade the host immune system, comprising of both the innate and adaptive immune responses. The innate immune system is the key mechanism of host resistance to many organisms, during the early stages of infection (Scharton-Kersten & Sher, 1997; Tiwari *et al.*, 2008). In 2004, Chang *et al.* found that during the early stages of adhesion, *T. vaginalis* rapidly activated nuclear factor-κB (NF-κB) in macrophages, resulting in the inhibition of pro-inflammatory cytokine production. It was therefore suggested, that the organism induces an inhibitory mechanism in order to evade host immunity (Chang *et al.*, 2004).

The secretion of large quantities of soluble antigens by *T. vaginalis* may prevent the direct attack of antibodies or lymphocytes on the organism (Mason & Patterson, 1985). *Trichomonas vaginalis* employs a phenotypic variation of P270, an immunogenic glycoprotein, as a mechanism of immune evasion. The organism may or may not express this immunogenic glycoprotein. The immunogenic glycoprotein P270 is alternately expressed with adhesins (AP65, AP51, AP33 and AP23) on the surface of the organism. *Trichomonas vaginalis* is therefore designated as P270+ for those organisms expressing the immunogen, and P270- for those lacking the immunogen. This antigenic variation could be an important step in host immune evasion. *In vitro,* the shift from a heterogeneous population to P270- organisms expressing the adhesions enables greater adherence to VECs (Alderete & Kasmala, 1986; Alderete *et al.*, 1986; Alderete, 1988).

"*Trichomonas vaginalis* activates the alternative pathway of complement" (Petrin *et al.*, 1998). Cervical mucus was found to be deficient in complement; therefore menstrual blood is the only source of vaginal complement (Alderete *et al.*, 1995d).

Iron was found to be a contributing factor in complement resistance. When grown *in vitro* in iron-enriched medium, *T. vaginalis* has been shown to be resistant to cell lysis via the alternative pathway of complement (Alderete *et al.*, 1995d). *In vivo*, lactoferrin, a principle source of iron for the organism, confers resistance to complement mediated cell lysis. It has been suggested, that lactoferrin is responsible for up-regulating expression of the gene, encoding the cysteine proteinases that degrade the C3 portion of complement (Alderete *et al.*, 1995d). During menses, when the microenvironment is saturated with erythrocytes, proteins, immunoglobulins and complement, symptoms of trichomoniasis are exacerbated. These erythrocytes provide *T. vaginalis* with an abundant source of iron. This results in the expression of cysteine proteinases, allowing the organism to evade complement destruction (Alderete *et al.*, 1995d).

In addition to C3, cysteine proteinases secreted by the organism degrades immunoglobulins IgG, IgM and IgA. This in turn, results in antibody destruction thus avoiding antibody attack (Hernandez-Gutierrez *et al.*, 2004).

2.7.3 Adherence and adhesins (contact dependent cytotoxicity)

A vital requirement of *T. vaginalis* for infection is the ability to adhere to VECs (Alderete & Garza, 1985). Cell attachment is time, temperature and pH dependent (Arroyo *et al.*, 1992). *Trichomonas vaginalis* not only interacts with VECs *in vitro*, but has also been observed to kill VECs (Krieger *et al.*, 1985b).

Successful colonization of the vagina by the parasite is a complex multi-step process, initiated by interactions with mucin, a large glycoprotein and major constituent of mucus (Lehker & Sweeney, 1999; Alderete *et al.*, 2002). Many factors of *T. vaginalis* are involved in breaching the mucous layer. Epithelial adherence is mediated by five adhesion proteins designated AP120, AP65, AP51, AP33, and AP23 (Garcia & Alderete, 2007), acting in a specific receptor-ligand fashion (Alderete *et al.*, 1995c; Alderete *et al.*, 2002). Gene expression is regulated at the transcriptional level by iron (Alderete *et al.*, 1995c; Alderete *et al.*, 1995d).

Trichomonas vaginalis requires high concentrations of iron for growth and multiplication (Alderete *et al.*, 2004). The binding of lactoferrin results in an increased uptake of iron, which regulates adherence (Lehker *et al.*, 1991; Lehker & Alderete, 1992). In 2004, Alderete *et al.* reported that heme not only aids multiplication in an iron-limiting medium, but also increases "transcription and surface placement of the AP65 adhesin", thereby enhancing adherence (Alderete *et al.*, 2004). Haemoglobin and lactoferrin receptors, involved in iron acquisition, are located on the surface of the parasite, and may contribute to the organism's survival in sites of different nutritional compositions within the host (Alderete *et al.*, 2004).

Recent findings illustrating the complexity of *T. vaginalis* in its interaction with fibronectin (FN) contributes to the understanding of the nature of infection. The parasite specifically binds to surface receptors by means of immobilized FN (Alderete *et al.*, 2002). The amount and affinity of bound FN is influenced by both iron and calcium concentrations (Crouch *et al.*, 2001; Alderete *et al.*, 2002). This indicates that depending on the iron status of the environment, calcium may influence recognition and binding to FN at sites of infection other than the surface epithelium. This mechanism may permit this organism to parasitize at multiple host sites (Crouch *et al.*, 2001).

Adhesin synthesis is up-regulated when the parasite comes in contact with a cell (Kucknoor *et al.*, 2005a). It has been reported, that the opposite side of the undulating membrane of the parasite harbors high concentrations of adhesins and microfilaments (Alderete & Garza, 1985; Rasmussen *et al.*, 1986). Therefore, this side is used for attachment to epithelial cells.

Another mechanism of pathogenesis is alternating surface expression of macromolecules (Alderete *et al.*, 2002). As discussed earlier, glycoprotein P270 is alternately expressed with adhesins on the surface of the parasite, and has been implicated with a variation in adhesion to epithelial cells (Alderete, 1988; Alderete & Garza, 1988; Alderete *et al.*, 1992). It was observed, that adherent negative organisms expressed P270 whereas adherent positive did not (Alderete & Garza, 1988).

Laminin, a glycoprotein restricted to the epithelial basement membrane, promotes cell adhesion (Costa e Silva Filho *et al.*, 1988). The parasite's receptors may play a role in pathogenesis. Unlike adhesins, laminin-binding proteins spans over the entire surface of the

parasite. *Trichomonas vaginalis* was found to adhere to laminin-coated plastic (Costa e Silva Filho *et al.*, 1988).

2.7.4 Haemolysis

Trichomonas vaginalis cannot synthesize de nova pyramidines, purines and fatty acids and therefore relies on its environment to supply these substances. *Trichomonas vaginalis* has the ability to lyse erythrocytes, thereby acquiring an ideal source of fatty acids (Tiwari *et al.*, 2008). Apart from lipids, iron is also acquired via haemolysis (Rosset *et al.*, 2002; Tiwari *et al.*, 2008).

Haemolysis is a three step process (Rosset *et al.*, 2002) incorporating adhesion molecules. Trichomonads attach to erythrocytes in a specific ligand-receptor fashion. This triggers the release of perforin-like proteins, such as cysteine proteinases, resulting in trichomonad detachment and erythrocyte lysis (Rosset *et al.*, 2002). *In vitro*, haemolysis occurs mostly at pH 4.5 suggesting the suitability of the normal vaginal milieu for such activity (Fiori *et al.*, 1993; Rosset *et al.*, 2002).

It has been reported, that the cell-free filtrates of *T. vaginalis* are capable of erythrocyte lysis (Pindak *et al.*, 1993). The cause of this effect was assumed to be the metabolic products of *T. vaginalis,* such as fatty acids. It was also established that as the pH increased above 5.0, the parasite was capable of erythrocyte lysis without direct contact with the target cells (Fiori *et al.*, 1996).

2.7.5 Proteinases

Trichomonas vaginalis contains several surface proteinases, reported to play an important role in pathogenesis (Hernandez-Gutierrez *et al.*, 2004). The isolation and purification of *T. vaginalis* proteinases have been achieved permitting study of these enzymes (Irvine *et al.*, 1993; Irvine *et al.*, 1997). *Trichomonas vaginalis* has been reported to have between 11 and 23 distinct cysteine proteinases, including those found on the cell surface and excreted by the parasite (Arroyo & Alderete, 1989; Provenzano & Alderete, 1995). Various cysteine proteinase inhibitors have been used in order to determine which cysteine proteinases are vital for the survival of the parasite (Irvine *et al.,* 1997). The reducing environment of the vagina provides a suitable milieu for the activation of cysteine proteinases (Alderete *et al.*, 1991a; Alderete *et al.*, 1991b).

Trichomonas vaginalis has been reported to employ capping in order to cleave host immunoglobulins (including IgG), laminin, and haemoglobin, with cysteine proteinases found in the supernatants of logarithmically growing cultures (Provenzano & Alderete, 1995; Ryu & Min, 2006). Since *T. vaginalis* proteinases are capable of degrading various substrates, they can also degrade mucin permitting the organism to traverse the mucous layer (Lehker & Sweeney, 1999).

In addition to this, cysteine proteinases found on the parasite's surface are a prerequisite for the adherence of *T. vaginalis* to VECs (Garber & Lemchuk-Favel, 1994). Pre-treating trichomonads with a cysteine proteinase inhibitor TLCK (N-alpha-p-tosyl-L-lysine chloromethyl ketone), decreased the parasite's ability to adhere to epithelial cells. Incubating TLCK-treated parasites with other cysteine proteinases restored the parasite's ability to adhere

(Arroyo & Alderete, 1989). Two surface proteinases CP65 and CP30, involved in cytoadherence, are active at the pH and temperatures found in the vagina during infection (Hernandez-Gutierrez *et al.*, 2004). Both CP65 and CP30 degrade vaginal proteins, such as FN. However, only CP30 degrades hemoglobin (Alvarez-Sanchez *et al.*, 2000; Mendoza-Lopez *et al.*, 2000; Hernandez-Gutierrez *et al.*, 2004). Hernandez-Gutierrez *et al.* (2004) reported that CP39 binds to VECs, but not to prostatic and colon cancer cells suggesting tissue-specific binding. Proteinase CP39 was found to degrade immunoglobulins, FN, as well as hemoglobin (Hernandez-Gutierrez *et al.*, 2004). It has also been suggested, that cysteine proteinases inhibit lysis via complement by degrading C3 when deposited on the parasite's surface (Alderete *et al.*, 1995d).

2.7.6 Cell detaching factor (CDF)

Although contact-dependent mechanisms have been shown to play important roles in pathogenesis, contact-independent mechanisms are also involved. These mechanisms were initially reported by Hogue in 1943, who studied the adverse effects cell-free filtrates of trichomonads had on cells in culture. Secreted in the cell-free filtrate was a soluble factor, named cell detaching factor (CDF) (Garber *et al.*, 1989; Garber & Lemchuk-Favel, 1994). This soluble factor was found to be a 200 kDa glycoprotein. This glycoprotein was reported to cause cytopathic effects, such as the detachment and clumping of the cell monolayer *in vitro* (Pindak *et al.*, 1986; Garber *et al.*, 1989). Cell detaching factor was also capable of inhibiting freshly added cells from monolayer formation (Pindak *et al.*, 1986). The *in vitro* detachment of cells is considered to be analogous to the shedding of VECs from the vaginal mucosa, during acute *T. vaginalis* infection (Garber *et al.*, 1989), implying that CDF may play a role in pathogenesis.

The CDF concentration in culture filtrates was found to fluctuate depending on three factors. These factors include the "duration of *T. vaginalis* growth prior to filtrate preparation, the initial inoculum of *T. vaginalis*, and pH of the filtrate prior to harvesting" (Garber *et al.*, 1989).

The optimum pH of the cell-free filtrate for CDF production was found to be around 7.2 (Pindak *et al.*, 1986). However, purified CDF displayed optimum activity at pH 6.5 (Garber *et al.*, 1989). Activity varied between pH 5.0 and pH 8.5 (Garber *et al.*, 1989). Cell detaching factor was also found to be inactive below pH 4.5. Clinically, this was important because the normal vaginal pH is 4.5 and during trichomoniasis the pH is greater than 5.0. This rise in vaginal pH during infection may be crucial in the pathogenesis of *T. vaginalis* (Petrin *et al.*, 1998).

There lies an association between CDF concentration and the severity of clinical vaginitis. However, it is uncertain whether the CDF level, its activity, its immunogenecity, or a combination of the three contributes to the severity of clinical vaginitis. In fact, all of the pathogenic mechanisms possibly contribute to the virulence of this organism. Estrogen levels have been found to affect CDF production. The severity of symptoms has been shown to increase during menses, when estrogen levels are the lowest (Garber *et al.*, 1991).

CDF seems to play a vital role in the pathogenesis of *T. vaginalis*. Therefore, understanding its function may help elucidate the organism's ability to invade, adjust, and adapt to a hostile and unreceptive female genital tract.

CHAPTER 3

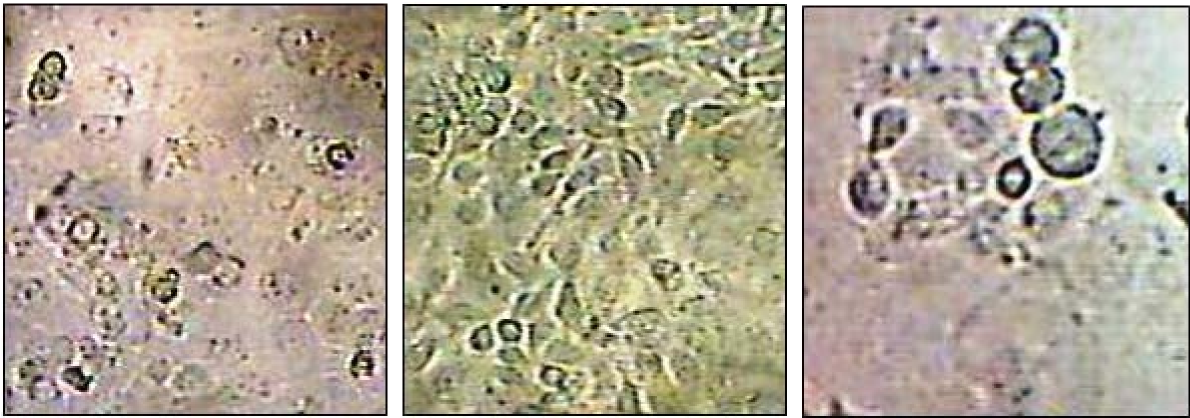
METHODOLOGY

3.1 Growth and maintenance of *Trichomonas vaginalis*

Trichomonas vaginalis isolates were obtained from patients enrolled in a concomitant study. Vaginal swabs were placed into Diamond's culture medium. Organisms were maintained as stock cultures for several weeks by passaging in 5mLs of Diamond's trypticase-yeast-extract-maltose medium (pH 6) supplemented with 10% heat inactivated horse serum with 4µg/mL amikacin, 1mg/mL ampicillin and 5mg/mL amphotericin B. Organisms used in the experiments were in the logarithmic growth phase. This was achieved by subculturing 500µl of *T. vaginalis* stock culture for 48hrs in 5mLs of Diamond's medium. Organisms were sub-cultured in agar free media a week before experiments in order to prevent clumping.

3.2 Cell lines

The three cell lines used in this study were purchased from the American Type Culture Collection (ATCC), Virginia (USA). The vaginal epithelial cell line VK2 (ATCC, catalogue number CRL-2616) was derived from normal vaginal mucosal tissue acquired from a 32 year old pre-menopausal woman undergoing vaginal repair surgery in 1996 (Fichorova & Anderson, 1999). The cervical epithelial cell line ME-180 (ATCC, catalogue number HTB-33) was derived from a highly invasive cervical squamous cell carcinoma of a 66 year old female. The endometrial cell line KLE (ATCC, catalogue number CRL-1622) was derived from a poorly differentiated endometrial adenocarcinoma of a 64 year old female. Figure 3.1 shows light microscopic (Nikon Diaphot, Carl Zeis (PTY) Limited) pictures of these three cell lines.



VK2 **ME-180** **KLE**

Figure 3.1 Vaginal (VK2), cervical (ME-180) and endometrial (KLE) cell lines used in experiments (40X)

The culture media used to propagate these cell lines differed due to differences in nutritional requirements between the cell lines. According to ATCC instructions, the VK2 cells required Keratinocyte-Serum free medium (GIBCO, catalogue number BRL 17005-042) supplemented with 0.1mg/mL human recombinant EGF, 0.05mg/mL bovine pituitary extract and additional calcium chloride with a final concentration of 0.4mM. The cervical epithelial cells were cultured in McCoy's 5a Medium containing 1.5mM L-glutamine and 2.2g/L sodium bicarbonate. The endometrial cells were grown in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium containing 2.5mM L-glutamine, 1.34g/L sodium bicarbonate and 2.25g/L glucose. The culture medium for both ME-180 and KLE was supplemented with heat-inactivated foetal calf serum (FCS).

3.2.1 Cell propagation

In resuscitating frozen cells, the cryovial containing the cells was thawed by rapid agitation in a water-bath at 37^0C. The cryovial was then removed from the water-bath and disinfected with 70% ethanol before transfer into the laminar flow cabinet. Cells were then transferred to a 15mLs conical tube, to which 10mLs of the appropriate fresh growth medium was added slowly as not to shock the cells. The cell suspension was then centrifuged (Heraeus multifuge 3S-R, 2006, Thermo Electron Corporation) at 1200rpm for 10min and the supernatant was discarded. This was done in order to rid the cells of preservatives, such as dimethyl-sulphoxide (DMSO) which is cytotoxic. The pellet of cells obtained was then re-suspended in 10mLs of fresh growth medium. The cell suspension was then seeded into equilibrated 75cc tissue culture treated flasks to which an additional 10mLs of fresh culture medium was added. The cells were then incubated at 37^0C in 5% CO_2 until a confluent monolayer was obtained. The incubation time varied between four to ten days, depending on the cell line. Cells were maintained every 48hrs with fresh maintenance medium.

According to the ATCC, the culture media for both KLE and VK2 differed however the media used for freezing and resuscitating these cell lines were the same, that being 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12, supplemented with 10% FCS, which deactivates the preservative. All cell lines were grown individually, preventing possible contamination. Cell growth was observed using an inverted microscope (Nikon Diaphot, Carl Zeis (PTY) Limited).

3.2.2 Cell counts

Cell counts were performed using the trypan blue exclusion method. One part of 1% trypan blue dye was gently added to one part of cell suspension. Only the dead cells stained blue, whereas the viable cells remain unstained.

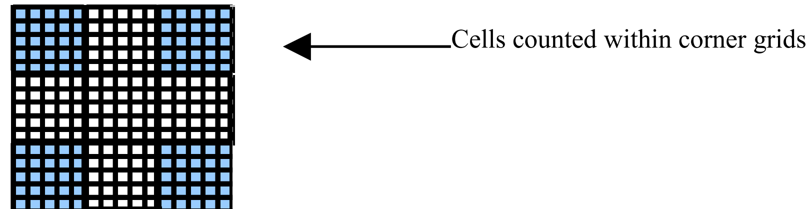


Figure 3.2 Counting grids of a Neubauer Haemocytometer

A Neubauer Haemocytometer was filled with 10µl of the cell-dye suspension and all the dead as well as the viable cells were counted as soon as the cells had settled.

The number of cells per mL was calculated using the following formula: -

$$\text{Number of cells} = \text{N x dilution factor x dilution volume}$$

$$N = \frac{\text{Number of viable cells counted}}{\text{Number of squares}} \times 2$$

The factor 2 corrects for the dilution with trypan blue.

Example: If you count 50 viable cells in the four corner squares shown in Figure 3.2, then,

$$\text{Number of cells} = \frac{50}{4} \times 2 \times 10^4 \text{ cells/mL}$$

$$= 25 \times 10^4 \text{ cells/mL}$$

$$= 2.5 \times 10^5 \text{ cells/mL}$$

The percentage viability was determined using the following formula: -

$$\text{\% cell viability} = \frac{\text{Number of viable cells counted}}{\text{Total number of cells counted}} \times 100$$

(Source: http://www.nuncbrand.com/en/frame.aspx?ID=1174)

3.2.3 Passaging of cells

Once attaining confluency, the monolayer was trypsinized using a combination of the proteolytic enzyme trypsin and ethylene-diamine-tetra-acetc acid (EDTA). Trypsin degrades the extracellular tissue protein matrix, which aids cells in adhering to each other. Ethylene-diamine-tetra-acetic acid is a chelating agent, removing the magnesium and calcium ions, which are needed for the attachment of cells to the plastic flask wall.

To achieve this, old medium was discarded and cells were washed three times with warm phosphate buffered saline (PBS). Pre-warmed trypsin-EDTA was added and dispersed evenly by gently rocking the flask. The flask was then incubated for 1 to 3min (depending on the cell line) at 37^0C. Trypsin is harmful to living cells; therefore cells were exposed for the shortest possible time. The flask was then tapped against the palm of the hand, to assist in cell detachment. The effect of trypsin was then counteracted by the addition of the respective growth medium supplemented with 10% FCS, as soon as the cells were detached.

Since VK2 cells grow in serum-free medium only, the cell suspension containing FCS was centrifuged. After which, the supernatant was discarded and the cells re-suspended in keratinocyte-serum free culture medium.

The cell lines were sub-cultured according to ATCC instructions. The cell concentration was adjusted to the desired concentration according to a predetermined ratio of 1:3 to 1:5 for VK2, 1:2 to1:3 for KLE and 1:3 to 1:8 for ME-180. To avoid loss of differentiation, these cell lines were not split at very high ratios. Cells were then seeded into new flasks, which were then

incubated in 5% CO_2 at 37^0C for 48hrs, during which these cells repaired their membranes and multiplied forming a monolayer.

3.2.4 Cryopreservation of cells

Freezing fluid containing 85% culture medium, 10% FCS and 5% DMSO was added to the cell suspension, slowly as not to shock the cells. This was followed by a step-wise process, during which, the cells were placed at 4^0C for one hour, -20^0C overnight and thereafter at -70^0C (for up to three months) or storing in liquid nitrogen at $–196^0$C.

3.3 The effect of pH on the growth and survival of *T. vaginalis*

In studying the effect pH has on *T. vaginalis*, portions of the Diamond's medium used was modified with 1N HCl and 1N NaOH so that the pH ranged from 4.5 to 7 in increments of 0.5. For the assay, 500µl of cultured *T. vaginalis* was added to 5mLs of modified Diamond's medium and incubated at 37^0C in 5% CO_2 for 48hrs. Cell counts were performed at 0, 24 and 48hrs intervals. The pH was recorded at 0, 4, 8, 24 and 48hrs intervals.

3.4 Binding medium

To be able to study the adhesion of the protozoa to the cells, a medium needed to be developed that supported both the parasites and the cell line used. This was achieved by mixing Diamond's medium with the cell specific tissue culture medium in a ratio of 1:2. This is referred to as "binding medium" (Alderete & Garza, 1985; Arroyo & Alderete, 1989).

3.5 Effects of environmental changes

Since the pH in the vagina changes during the menstrual cycle, adhesion of trichomonads was studied at different pH reflecting these changes. Portions of the binding medium were modified with 1N HCl and 1N NaOH so that the pH ranged from 4.5 to 7 in increments of 0.5.

In addition, we studied the difference in adhesion at surface (33^0C) and core (37^0C) body temperature. All tests were done three times in triplicate.

3.6 Adhesion assay

In developing an adhesion assay (Alderete & Garza, 1985; Arroyo & Alderete, 1989), cells were seeded on 12mm glass coverslips in the wells of 24 well micotiter plates. These were incubated in 5% CO_2 at 37^0C until reaching 90% confluency. The culture medium was then aspirated and cells were washed three times with 2mls of warm sterile PBS. Following this, 1ml of parasite suspension (in modified binding medium containing the predetermined number of trichomonads) was added to the confluent cell monolayers. Cells and trichomonads were then co-incubated for 1hr in 5% CO_2 at the required temperature. After incubation, unbound trichomonads were removed by washing three times with 2mls of warm sterile PBS. The coverslips were removed, stained with acridine orange and viewed under a fluorescent microscope (DF Nikon Labophot, Carl Zeiss (PTY) Limited). The number of adhered trichomonads per 100 cells was counted.

3.7 Cytotoxicity studies

Cytotoxicity assays were done in the wells of a Costar 96 well tissue culture treated microtiter plate, in triplicate as shown in Figure 3.4. Cells were seeded into individual wells and incubated in 5% CO_2 at 37^0C until reaching 90% confluency. Empty wells were filled with sterilized water, in order to maintain moisture. Cell counts were performed for each cell line, in order to determine the inoculum. Prior to inoculation, the cell culture medium was aspirated and cells were washed three times with warm sterile PBS. Following this, 100µl of parasite suspension in binding medium with different pH was added to the cell monolayers, at the desired multiplication of infection (MOI). The inoculated monolayers were incubated for 6hrs in 5% CO_2 at 37^0C. Control wells contained cells only. Cytotoxicity was then quantitatively measured using the non-radioactive Cytotox 96® assay kit from Promega (Bastida-Corcuera *et al.*, 2005).

The Cytotox 96® Assay quantitatively measures lactate dehydrogenase (LDH), a cytosolic enzyme that is released upon cell lysis. The amount of LDH released in culture supernatants was measured with a 30mins enzymatic assay in which LDH converts tetrazolium salt (INT) to red formazan. The color intensity is proportional to the amount of released LDH and through that, to the number of lysed cells. This was measured by means of spectrophotometry at 490nm on a 96 well plate reader (Anthos Reader, 2001, SepSci Technical Services Ltd). Figure 3.3 shows the 96 well assay plate layouts as used for each cell line at different pH (4.5 to 7), with the contents of the different wells shown in different colours.

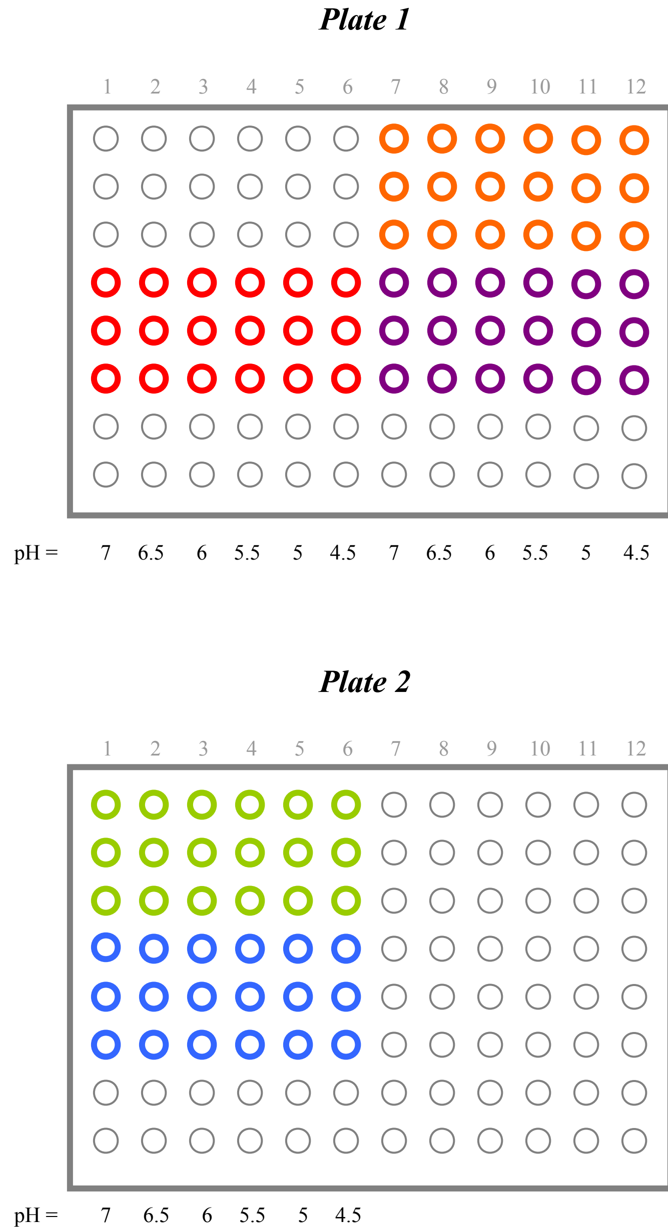


Figure 3.3 Lay-out of the cytotoxicity assay plates. Red: wells with trichomonads (effector) only. Blue: wells with cells (target) only. Green: Maximum (100%) release of LDH by cells only. Orange: Volume correction control, which corrects for the volume change caused by adding lyses solution. Purple: culture medium background, which corrects for LDH activity contributed by both the serum and the varying amounts of phenol red in the culture medium.

Forty five minutes prior to the end of the incubation time, 10µl of lysis solution was added for every 100µl of target cells to the wells marked as Target Cell Maximum LDH Release Control. After incubation, the plates were centrifuged (Heraeus multifuge 3S-R, 2006, Thermo Electron Corporation) at 4000rpm for 4min.

In measuring the LDH concentration, 50µl aliquots of supernatant from all wells were transferred to a fresh 96 well enzyme assay plate, to which 50µl of Substrate Mix was added. The plate was then covered with foil, to protect it from light, and incubated for 30mins at room temperature. Thereafter, 50µl of Stop Solution was added to each well and absorbance values were recorded within 1hr. All reagents were provided with the kit. The following formula was used to calculate the percentage of cytotoxicity:

$$\frac{\text{experimental} - \text{effector spontaneous} - \text{target spontaneous}}{\text{target maximum} - \text{target spontaneous}} \times 100$$

(effector = trichomonads; target = tissue culture cells)

(Source: http://www.promega.com/catalog/category.aspx?catergoryname=productgroup_39)

3.8 Data analysis

Trend analysis and statistical significance was determined using SPSS version 15.0 (SPSS Inc., Chicago, Illinois). A p value <0.05 was considered as statistically significant. Multiple comparisons between each pH of each cell line were done. Dependant variables were tested using histograms and the skewness statistic.

3.9 Ethical consideration

This study was approved by the Faculty of Medicine Ethics Committee, E154/03.

CHAPTER 4

RESULTS

4.1 Growth and Survival of *T. vaginalis* at different pH values

The effect of pH on the growth and survival of *T. vaginalis* is summarised in Figure 4.1. At 48hrs, the concentration of *T. vaginalis* increased sharply between pH 5.5 and 7 with the maximum number of viable organisms between pH 6 and 6.5. Most of the organisms in the first 24hrs at pH 4.5 had died, thus indicating the importance of environmental pH. There was also a marked increase of pH 5 and pH 5.5 to pH 6 at 24hrs.

In analyzing these growth and survival data, more than twice the standard error of skewness indicated significant skewness. The dependant variables were therefore log-transformed due to abnormality. Two-way ANOVA testing was used to assess the full factorial model. Both dependent variables, time and pH were found to be statistically significant ($p<0.001$).

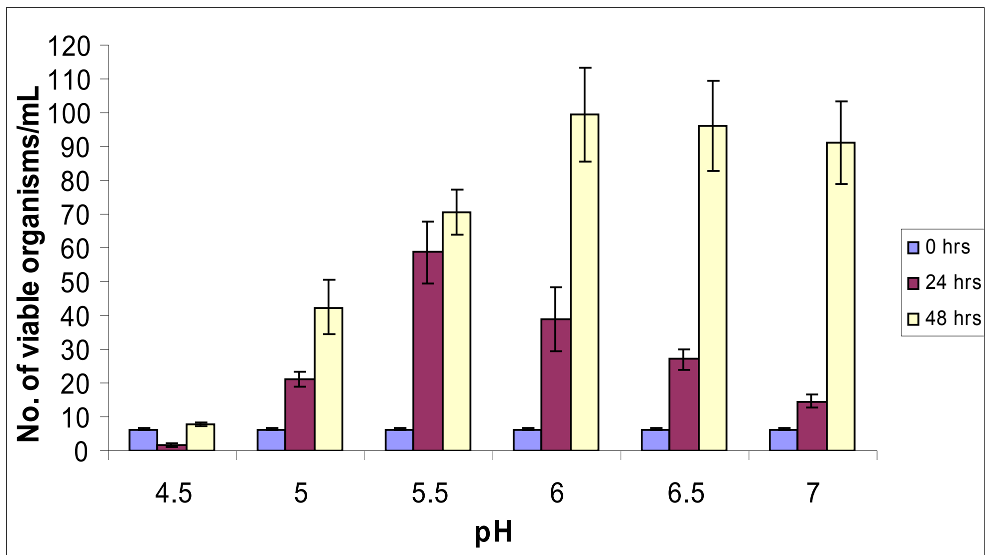


Figure 4.1 Growth and survival results of *T. vaginalis* sub-cultured in media of different pH (4.5 to 7) over a 48hrs period. The number of viable organisms was counted at 0, 24 and 48hrs, $p<0.001$.

4.2 Adherence of *T. vaginalis* to cell monolayers

Figure 4.2 shows acridine orange stained cells exposed to *T. vaginalis*.

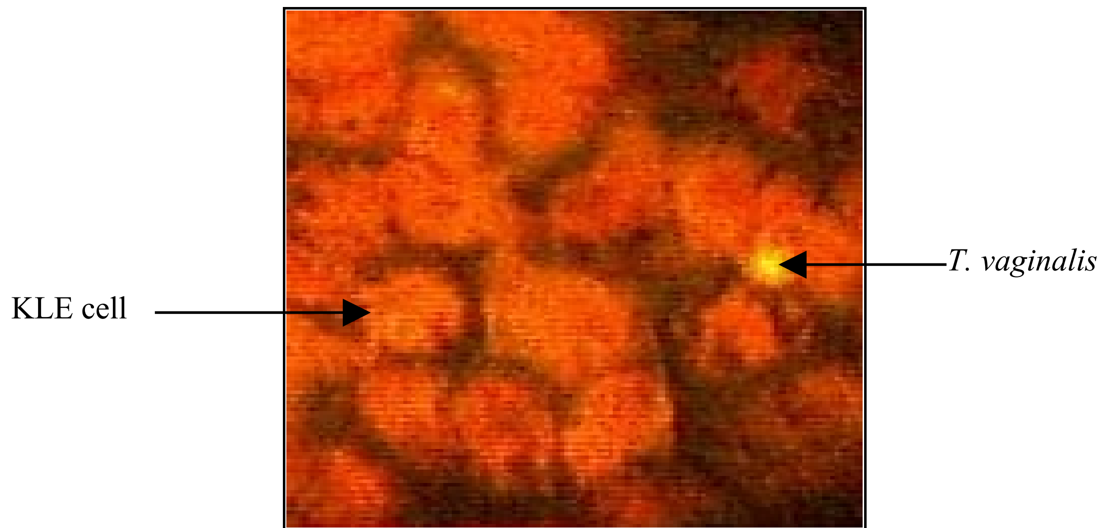


Figure 4.2 Acridine orange stain of the KLE cell line with adhered *T. vaginalis* (40X). *T. vaginalis* can be easily distinguished against the dark orange background of cells.

The inter- and intra-test variation for the adhesion assays for all three cell lines at 37^0C and 33^0C is shown in Tables 4.1 and 4.2. The percentage adherence of *T. vaginalis* to the different cell lines is summarized in Figures 4.3 to 4.8.

Table 4.1: Triplicate values of adherent trichomonads for three tests done on different days at 37^0C.

pH	Cell line	Test 1			Test 2			Test 3		
pH7	VK2	10.5	8.5	9	8.5	7.5	8	5.5	6.5	5.5
pH6.5	VK2	8	7.5	7.5	6	6.5	6	5	5.5	4.5
pH6	VK2	6	5.5	5.5	4.5	5.5	5	5	4.5	4
pH5.5	VK2	5	4	5.5	4.5	5	4	4.5	4.5	4
pH5	VK2	8	5.5	6.5	6	5.5	5.5	6	5.5	5.5
pH4.5	VK2	4.5	4.5	4.5	4.5	4	4	4	3.5	4
pH7	ME-180	8	7	7	7	6	5.5	5	4.5	4.5
pH6.5	ME-180	9.5	8	8.5	7	7	7.5	6	6.5	5.5
pH6	ME-180	4	4	4.5	3.5	4	4	3.5	3	3
pH5.5	ME-180	4.5	5	4	4	4	3.5	4	3.5	3.5
pH5	ME-180	2.5	4	2	2.5	2	2.5	2.5	2	3
pH4.5	ME-180	1	1.5	1.5	2	2	2	2	1.5	1.5
pH7	KLE	6	5	4.5	5.5	4.5	4	4.5	4	4
pH6.5	KLE	6	7.5	5.5	5.5	5	5	4.5	4.5	4
pH6	KLE	8	9	8	7.5	8	7.5	7	7	7.5
pH5.5	KLE	5	6.5	5	5.5	5.5	4.5	5	5.5	5
pH5	KLE	9.5	6	10	8.5	8	7.5	8	8	8
pH4.5	KLE	9	7.5	9.5	9	8	8	8.5	8.5	8

Table 4.2: Triplicate values of adherent trichomonads for three tests done on different days at 33^0C.

pH	Cell line	Test 1			Test 2			Test 3		
pH7	VK2	7.5	7	8	7.5	7	8	7.5	7	8
pH6.5	VK2	8	8.5	8	8	8.5	8	8	8.5	8
pH6	VK2	8.5	8.5	9	8.5	8.5	9	8.5	8.5	9
pH5.5	VK2	11	10.5	10	11	10.5	10	11	10.5	10
pH5	VK2	12.5	13.5	14	12.5	13.5	14	12.5	13.5	14
pH4.5	VK2	13.5	13.5	14	13.5	13.5	14	13.5	13.5	14
pH7	ME-180	6.5	6.5	7	6.5	6.5	7	6.5	6.5	7
pH6.5	ME-180	8	8	7.5	8	8	7.5	8	8	7.5
pH6	ME-180	7.5	8	7.5	7.5	8	7.5	7.5	8	7.5
pH5.5	ME-180	14	13	13	14	13	13	14	13	13
pH5	ME-180	19.5	19.5	19	19.5	19.5	19	19.5	19.5	19
pH4.5	ME-180	20.5	20	20.5	20.5	20	20.5	20.5	20	20.5
pH7	KLE	3.5	4	4	3.5	4	4	3.5	4	4
pH6.5	KLE	3	3	2.5	3	3	2.5	3	3	2.5
pH6	KLE	1	2	1.5	1	2	1.5	1	2	1.5
pH5.5	KLE	1.5	1	1.5	1.5	1	1.5	1.5	1	1.5
pH5	KLE	1.5	1.5	1	1.5	1.5	1	1.5	1.5	1
pH4.5	KLE	3	2.5	2.5	3	2.5	2.5	3	2.5	2.5

Adherence to VK2 cells at 37^0C varied from an average of 4.2 trichomonads/100 cells at pH 4.5 to 7.7/100 cells at pH 7. At 33^0C these values were 13.7/100 cells and 7.5/100 cells respectively. For ME-180 cells the average number of adherent trichomonads at 37^0C was 1.7/100 cells at pH 4.5 and 6.1/100 cells at pH 7. At 33^0C the averages were 20.3/100 cells and 6.7/100 cells at the respective pH values. Adherence to KLE cells at 37^0C varied from an average of 8.4 trichomonads/100 cells at pH 4.5 to 4.7/100 cells at pH 7. At 33^0C these values were 2.7/100 cells and 3.8/100 cells respectively.

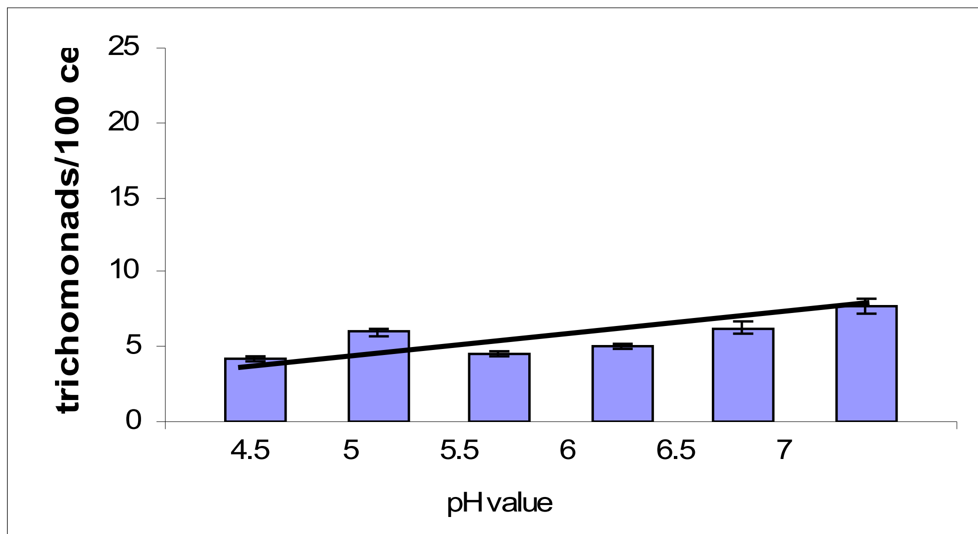


Figure 4.3 Adhesion assay results displaying the average number of trichomonads adhered to the VK2 cell line between pH 4.5 to 7 at 37^0C, $p<0.001$.

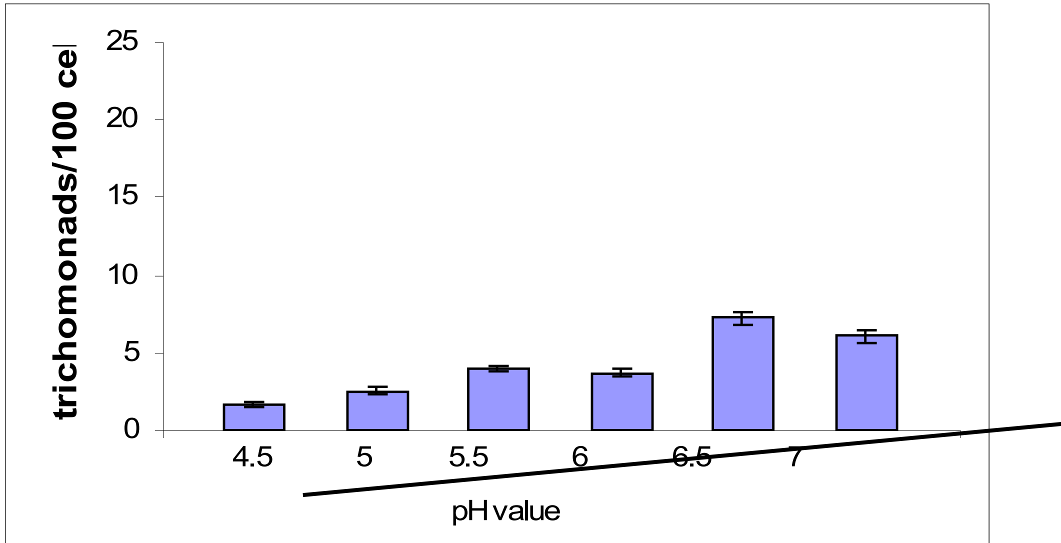


Figure 4.4 Adhesion assay results displaying the average number of trichomonads adhered to the ME-180 cell line between pH 4.5 to 7 at 37^0C, $p<0.001$.

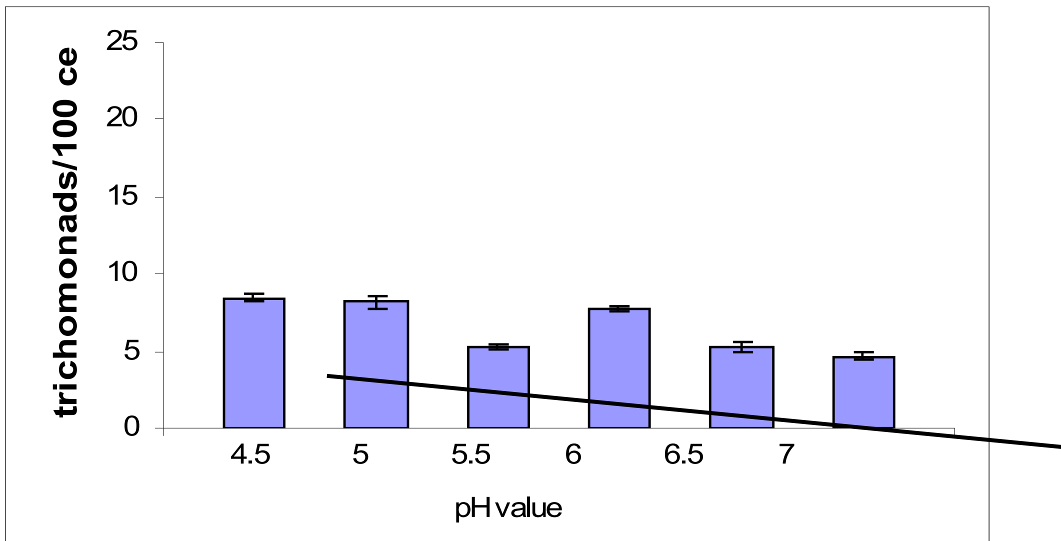


Figure 4.5 Adhesion assay results displaying the average number of trichomonads adhered to the KLE cell line between pH 4.5 to 7 at 37^0C, $p<0.001$.

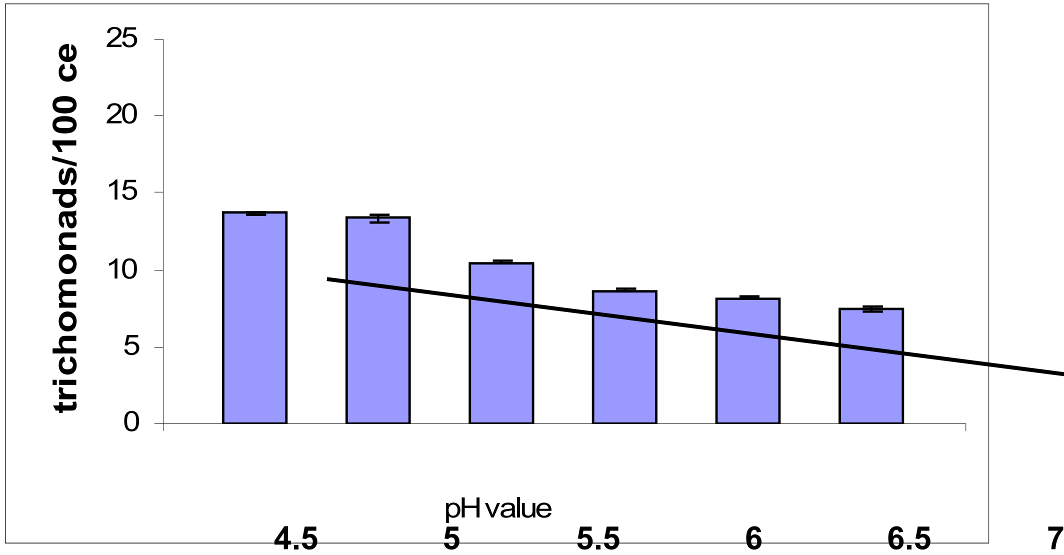


Figure 4.6 Adhesion assay results displaying the average number of trichomonads adhered to the VK2 cell line between pH 4.5 to 7 at 33^0C, $p<0.001$.

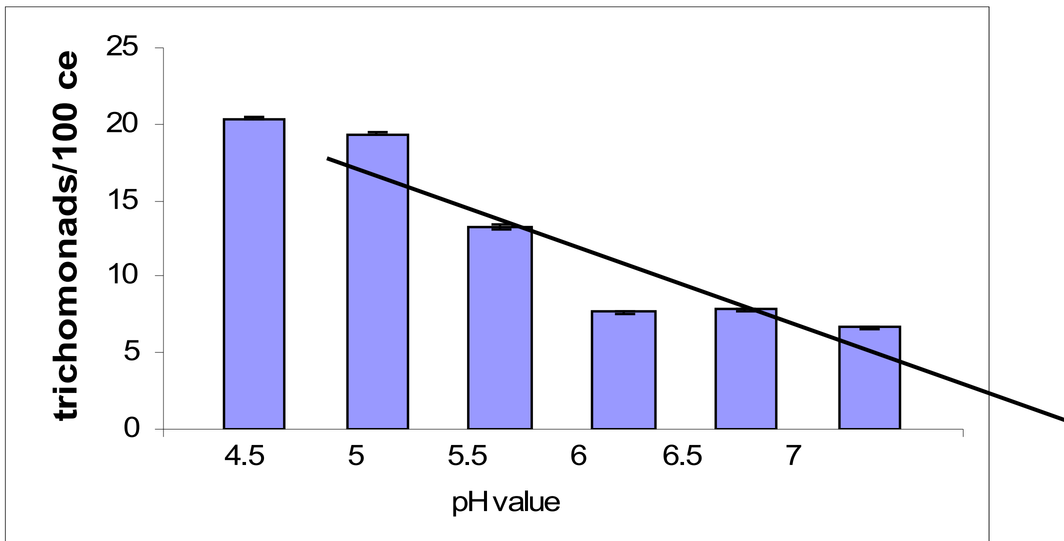


Figure 4.7 Adhesion assay results displaying the average number of trichomonads adhered to the ME-180 cell line between pH 4.5 to 7 at 33^0C, $p<0.001$.

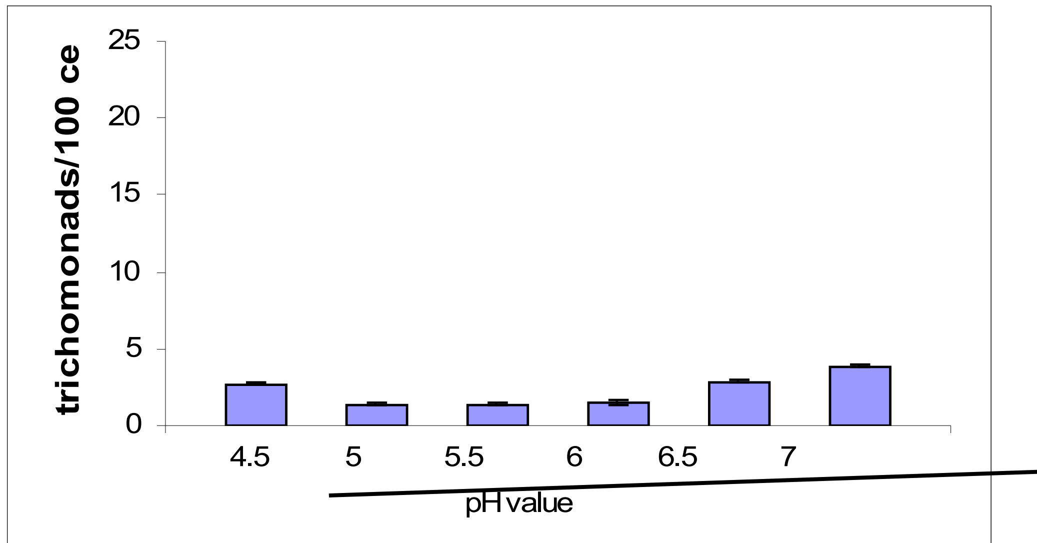


Figure 4.8 Adhesion assay results displaying the average number of trichomonads adhered to the KLE cell line between pH 4.5 to 7 at 33^{0}C, p<0.001.

There was an overall significant difference between the cell lines (p<0.001). While at 37^{0}C for both the VK2 and ME-180 cells adherence levels increased with rising pH, with the KLE cells the opposite trend was observed. At 33^{0}C, adherence to both the vaginal and cervical cells decreased at a higher rate with rising pH. The adherence to the KLE cell line at 33^{0}C decreased. The comparison of mean adhesion at the two temperatures was found to be significantly different (p=0.009). Adhesion at 33^{0}C was higher as compared to 37^{0}C for both the VK2 and ME-180 cells but not for the KLE cells. There was a higher significant interaction between all three variables (p<0.001). Therefore the effect of each factor depends on the effects of the other factors. The cell lines behave differently over the different pH values and the different temperatures. Overall, all three variables (cell line, temperature and pH) significantly affects adhesion.

4.3 Cytotoxicity

4.3.1 Microscopic observations

After 30mins post-inoculation, most trichomonads settled on the cell monolayers. After 2hrs they lay motionless, adhered to the cell monolayers. Eight hours later approximately 10% of the cell monolayers were destroyed. Cells that were free of the trichomonads lay next to those already destroyed indicating the importance of contact between the parasite and the host cell. Extended exposure of the cell monolayers to live trichomonads resulted in total loss of cell viability. The cytotoxicity of *T. vaginalis* is therefore a slow process which requires several hours of contact with the cell monolayers (Gilbert *et al.*, 2000).

4.3.2 LDH release assay

The percentage cell death was measured by means of the LDH release assay. The results for each cell line are given in Table 4.3 and are summarized in Figures 4.9 to 4.11.

Table 4.3: Percentage LDH release by different cell lines for the three experiments done on different days.

Cell line	pH	Test 1	Test 2	Test 3
VK2	4.5	10.71	15.48	1.99
VK2	5	52.51	38.99	55.21
VK2	5.5	55.92	61.22	64.08
VK2	6	32.79	28.19	32.87
VK2	6.5	50.92	53.37	41.1
VK2	7	12.97	23.27	31.26
ME-180	4.5	60.48	84.75	55.4
ME-180	5	40.64	33.56	38.54
ME-180	5.5	42.74	43.63	41.68
ME-180	6	29.72	29.65	30.14
ME-180	6.5	7.49	7.11	9.32
ME-180	7	8.19	7.24	9.24
KLE	4.5	-11.62	-10.42	-1.42
KLE	5	25.52	47.93	94.99
KLE	5.5	6.08	21.96	29.37
KLE	6	-4.88	0.82	10.43
KLE	6.5	3.87	0.44	13.29
KLE	7	14.03	9.31	15.81

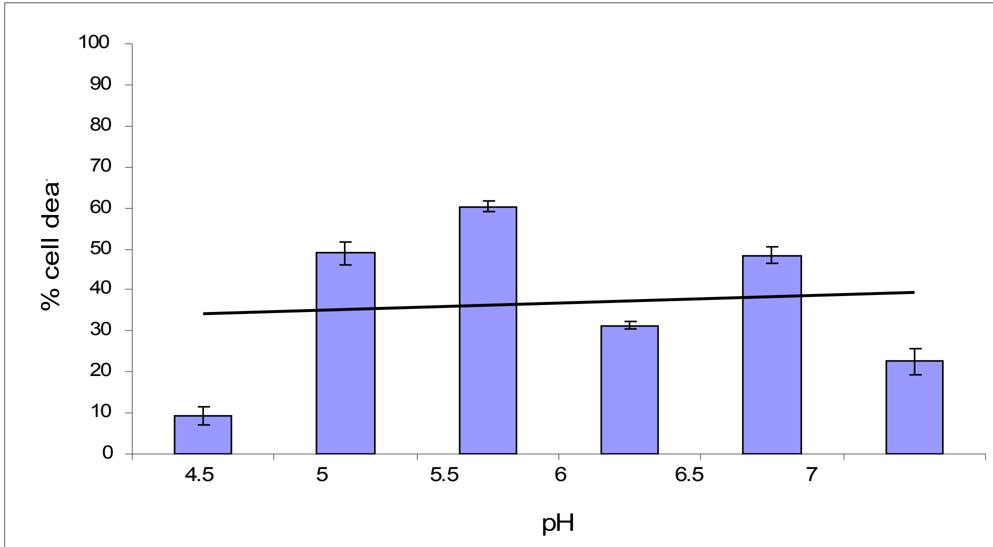


Figure 4.9 Cytotoxicity assay results displaying the average toxicity values of *T. vaginalis* on the VK2 cell line between pH 4.5 to 7 at 37^0C, $p<0.001$.

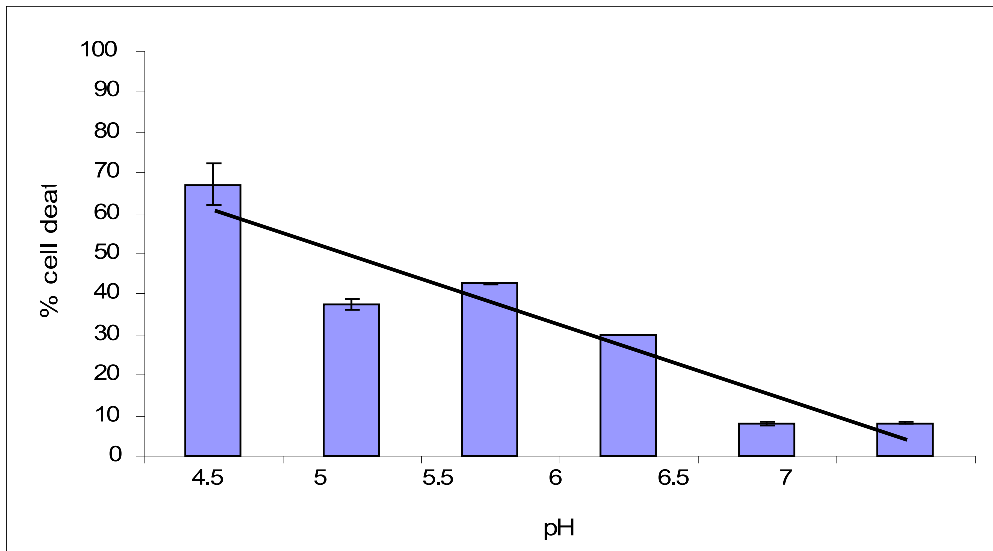


Figure 4.10 Cytotoxicity assay results displaying the average toxicity values of *T. vaginalis* on the ME-180 cell line between pH 4.5 to 7 at 37^0C, $p<0.001$.

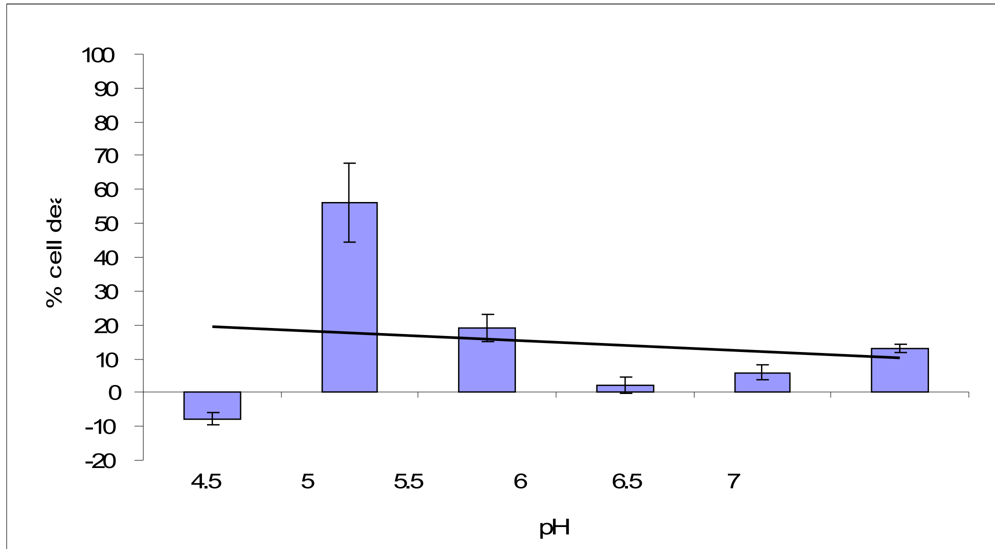


Figure 4.11 Cytotoxicity assay results displaying the average toxicity values of *T. vaginalis* on the KLE cell line between pH 4.5 to 7 at 37^{0}C, p<0.001.

The comparison of mean cytotoxicity values between the cell lines had a highly significant overall difference (p<0.001). The difference lay between ME-180 and each of the other two cell lines (p<0.001). The VK2 and KLE cell lines were not different from each other (p=0.012). The rate of change over pH between the cell lines differed significantly (p<0.001). The cytotoxicity of *T. vaginalis* to the ME-180 and KLE cell lines decreased with rising pH. The VK2 cell line was not affected by pH, although there was a sharp increase in cell death at the lower pH (4.5 to 5) and thereafter stabilizes. The interaction between all three variables was highly significant (p<0.001).

CHAPTER 5

DISCUSSION

For many years, the question regarding *T. vaginalis* involvement in infections of the female upper genital tract remained unresolved. This study aimed at contributing to the resolution of whether *T. vaginalis* is able to adhere to cells of the endometrium, and if this differs from adhesion to cells of the vagina and cervix. In addition, toxicity to these different cell types was compared. So far, the ability to adhere to vaginal epithelial cells, the cytotoxic effect on those cells and proteinase activity has been proposed as factors involved in the pathogenecity of trichomoniasis (Ryu & Min, 2006).

A vaginal infection with *T. vaginalis* usually starts when the pH is at its physiological value of 4.5 but during progression of the infection, the pH increases (Petrin *et al.*, 1998; Adegbaju & Morenikeji, 2008). Before embarking on studies of interaction between the parasites and the different host cell types, the viability and growth of the organism at different pH values was investigated. There seem to be two subpopulations of parasite cells. The majority died quickly at pH 4.5 but survival and growth rates improve with increasing pH. This confirms the need for the organisms to adjust the pH in the vagina to establish infection. Kostara *et al.* found that growth rate of *T. vaginalis* was pH dependent. A higher pH delayed the initiation of growth whereas a lower pH favoured growth and survival in the early decline phase (Kostara *et al.*, 1998).

Adherence is a pre-requisite to establish and in many infections to maintain an infection. Experiments exposing immortalised host cell lines to microbes have been employed to study this phenomenon (Garcia *et al.*, 2003; Bastida-Corcuera *et al*., 2005). Many of these experiments were done with cells either from non-human origin or those originating from an anatomical site not related to the site where the organism resides when causing disease. In

addition, most of these cell lines had lost their typical characteristics, which they expressed *in vivo* during the immortalisation process. Often, the type of culture strains used might have adjusted to growth *in vitro* and therefore, had lost important characteristics. In this study, cell lines were used from the anatomical sites that potentially can be infected with *T. vaginalis*. The organisms used were fresh clinical isolates and were therefore not the same in all the experiments. This is because *T. vaginalis* does not survive long in culture media and can only be passaged for a limited number of times. This might in part be responsible for variation in the observations. However, sub-culturing of trichomonads *in vitro* may affect the virulence of the pathogen. Trichomonads sub-cultured for several weeks were shown to synthesize lower amounts of adhesion proteins (Garcia & Alderete, 2007) resulting in possible loss of virulence.

Trichomonas vaginalis produces five surface proteins involved in adherence to human vaginal epithelial cells (VECs). These are named Adhesin Protein (AP) 120, AP65, AP51, AP33, and AP23. Protein AP65 is the most prominent adhesin of *T. vaginalis* (Garcia *et al.*, 2003) and appears to play a role in establishing infection. In 2004, Mundodi *et al.* found that down regulating the expression of AP65 decreases adherence of *T. vaginalis* to VECs (Mundodi *et al.*, 2004). Therefore, the characterization of this adhesin may lead to possible future intervention strategies.

An infection with *T. vaginalis* in the vagina involves detachment of cells from the vaginal wall known as sloughing (Petrin *et al*., 1998). *In vitro* detachment of cells from solid phase on which they grow is observed almost invariably on exposure to extra-cellular microbes. The question of whether this is equivalent to the *in vivo* phenomenon of sloughing cannot be answered.

This study focused on the differences in adhesion and toxicity to vaginal, cervical and endometrial cells. The main aim was to establish whether they can adhere and damage endometrial cells to at least the same extend as the other cells which are known to be affected by the parasite *in vivo*. This was done under different circumstances including the pH range found in the vagina when the infection develops as well as temperatures reflecting the difference between the surface temperature of the body and the core temperature.

The adhesion to vaginal and cervical epithelial cells was similar with a trend of decreased adhesion at higher pH. On the other hand, adhesion to the endometrial cells was not influenced by pH. However, the number of adhering trichomonads per 100 cells tended to be higher for endometrial cells as compared to the other two cell types (no literature is available to confirm this).

Regarding cytotoxicity, the highest level for cells of the lower genital tract was found at the lowest pH, in particular on cervical cells. This suggests rapid damage shortly after acquisition of the infection. This in turn, assists in neutralising the pH creating optimal circumstances for adhesion and multiplication (no literature is available to confirm this).

In 2002, Moodley *et al.* reported an association with *T. vaginalis* and PID in HIV positive women but not in HIV negative women (Moodley *et al.*, 2002). They speculated about the pathophysiology of this phenomenon. *Trichomonas vaginalis* could invade the upper genital tract in a higher percentage of HIV infected women because of the evolving immune incompetence. Although the CD4 counts of the women involved were not known, it has been

well established for tuberculosis that susceptibility to infection increases at CD4 counts far above those associated with immune suppression (Grant *et al*., 2007). The other option is that HIV infection enhances the penetrability of the cervical mucus plug as initiated by mucinases produced by *T. vaginalis*. This in turn, allows ascending infection by *T. vaginalis* with bacteria present in the vagina or by the bacteria only. The observation of this study adds value to the last option. The protozooan adheres in at least the same quantities to endometrial cells as to cells of the lower genital tract and exerts cytotoxicity, especially at pH 5 to 6. This indicates that *T. vaginalis* may be directly involved in infection of the upper genital tract. The inflammatory response to bacterial co-infection could be lowering the pH to levels that optimize the toxic effect of *T. vaginalis* (no literature is available to confirm this)..

Regarding pathogenesis of trichomoniasis, the observations of this study remains speculative and does not explain the role of HIV. Although observations *in vivo* are difficult to obtain, these are needed to understand this fully. Adhesion and cytotoxicity studies provide insight for studying the pathogenesis of *T. vaginalis* hopefully leading to therapeutic treatment and the prevention of trichomonal infections.

In conclusion, adhesion of trichomonads to the endometrial cell line suggests that *T. vaginalis* is capable of colonization of the upper genital tract. At pH values applicable to *in vivo* situation, toxicity was very low.

CHAPTER 6

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CHAPTER 7

APPENDICES

APPENDIX I –*T. VAGINALIS* AND TISSUE CULTURE MEDIA

1.1 Diamond's trypticase-yeast-extract-maltose medium

Trypticase	20g
Yeast extract	10g
* Glucose	10g
* Maltose	5g
* Agar	0.5g
L. cysteine	1g
L. ascorbic acid	0.2g
Distilled water (DH$_2$O)	900mL

* For agar free media, maltose and agar were substituted with glucose.

Dry ingredients were weighed out and dissolved in DH$_2$O. The media was adjusted with 1N HCl and 1N NaOH to pH 6 and autoclaved for 10mins. Once cooled, 100mL of heat inactivated horse serum and antibiotics (4µg/mL amikacin, 1mg/mL ampicillin and 5mg/mL amphotericin B) were added.

1.2 Antimicrobial solution

Amikacin	4µg/mL
Ampicillin	1mg/mL
Amphotericin B	5mg/mL

Ampicillin and amphotericin B were dissolved in 4mLs and 10mLs of sterile DH$_2$O respectively. One milliliter aliquots of each antibiotic was stored at -20^{0}C.

1.3 Horse serum and Foetal calf serum (FCS)

Horse serum and foetal calf serum was used in Diamond's and cell culture media respectively. These were heat inactivated by de-complementing at 56^0C for 1hr. This was then filter sterilized, aliquoted into 10mL tubes and stored at -20^0C. Quality control was carried out by incubating 5mLs of serum at 37^0C for one week.

APPENDIX II – SOLUTIONS AND REAGENTS

2.1 0.025% Trypsin-EDTA

0.05% EDTA (0.05g EDTA was dissolved in 100mLs PBS and filter sterilized)

0.25% Trypsin

PBS (pH 7)

EDTA was diluted in a ratio of 1:1 in PBS. A mixture of 1mL trypsin and 4mLs of diluted EDTA was filter sterilized, aliquoted in 2mLs eppendorfs and stored at -20^0C.

2.2 Acridine orange stain

0.2g Acridine orange powder

20mL of DH_2O

0.2M Acetate buffer (made up of 0.2M acetic acid and 0.2M sodium acetate, pH 4)

Stock solution was made up by dissolving acridine powder in DH_2O. This was aliquoted in 20mLs tubes, covered with foil and stored at 4^0C. Working solutions containing 0.5mL stock solution and 5mLs 0.2M acetate buffer was made fresh daily. Cover slips were air dried, heat fixed and flooded with acridine orange stain for 2mins. After 2mins, coverslips were rinsed with tap water and allowed to air dry.

2.3 Non-radioactive Cytotox 96® assay kit from Promega

Substrate mix (powder)

Assay buffer

LDH positive control

Lysis solution (10X)

Stop solution

The following controls were performed with the Cytotox 96® assay: -

1. Effector Cell Spontaneous LDH Release: corrects for the spontaneous release of LDH from effector cells (trichomonads).
2. Target Cell Spontaneous LDH Release: corrects for spontaneous release of LDH from target cells (tissue culture cells).
3. Target Cell Maximum LDH Release: required in calculations to determine 100% release of LDH.
4. Volume Correction Control: corrects for the volume change caused by adding lysis solution.
5. Culture Medium Background: corrects for LDH activity contributed by the serum in culture medium and the varying amounts of phenol red in the culture medium.

The Substrate Mix was dissolved in 12mLs of warm Assay Buffer. The Substrate Mix was protected from light and stored at -20^{0}C for ≤6 to 8 weeks. One bottle of reconstituted Substrate Mix was enough for two 96 well plates.

(Source: http://www.promega.com/catalog/category.aspx?categoryname=productgroup_39)