CHAPTER 1

INTRODUCTION

1.1 Background of study

Parasites are defined as any organism that derive key components for survival by inhabiting hosts like animals, plants or humans (Northrop-Clewes & Shaw, 2000; CDC, 2010). Parasites can be harmful to the host since they extract nutrients by infecting cells, organs as well multiplying within the host's tissues to continue living and hence limiting the growth rate of the host (Northrop-Clewes & Shaw, 2000). Some examples of parasites include *Ascaris lumbriocoides, Trichuris trichiura, Sparganum mansoni, Fasciola hepatica, Diphyllobothrium latum,* and others that can potentially infect most of the living things (Northrop-Clewes & Shaw, 2000).

In humans, parasitic infections can be considered dangerous as they are known to cause mild to serious illness which at times can lead to fatality (Northrop-Clewes & Shaw, 2000; WHO, 2005). Continuous threats to human health due to emerging or re-emerging parasitic infections are not only reported among rural populations but also in urban populations where millions of people are killed annually (Northrop-Clewes & Shaw, 2000; WHO, 2005). Three main classes of parasites that are capable of triggering diseases in humans are protozoa (i.e. Sarcodina, Mastigophora, Ciliphora and Sporozoa) helminthes (i.e. flatworms, thorny-headed worms and roundworms) and ectoparasites (i.e. arthropods) (CDC, 2010). Examples of parasitic diseases, the damage caused to humans and their causative agents that are considered harmful are summarized in Table 1.1 (WHO, 2005; Malla & Goyal, 2012).

Table 1.1: Lists of parasitic diseases and their descriptions

Parasitic diseases	**Descriptions**
Amoebiasis	Disruption of the intestinal wall leading to ulcerating mucosal lesions by the protozoan parasite *Entemoeba histolytica.*
Hookworm disease	Causes anaemia and protein malnutrition due to infection by soil-transmitted helminth such as *Necator americanus* and *Ancylostoma duodenale.*
Leishmaniasis	Infection by several species of flagellated protozoan parasites known as *Leishmania* spp. causing serious disfigurement as well as death.
Malaria	Disease is caused by four species of the protozoan parasite *Plasmodium* leaving the infected patients with acute renal failure, cerebral malaria, pulmonary oedema and death to some extent.
Schistosomiasis	Also known as bilharziasis that is associated with bladder cancer or renal failure by *Schistosoma haematobium* and liver fibrosis and portal hypertension by *S. mansoni*.
Trichomoniasis	Sexually transmitted disease caused by the protozoa *Trichomonas vaginalis*

Sexually transmitted diseases (STDs) as defined by Malla and Goyal (2012) are diseases attacking human as a result of sexual contacts and are transmitted through infections by pathogens such as bacteria, fungi, viruses, parasites and ectoparasites. Among these Sexually Transmitted Infections (STIs), parasitic infections are progressively gaining attention due to the significant health implications it has on humans (Malla & Goyal, 2012). Based on the listed parasitic diseases in human (Table 1.1), trichomoniasis or also known as "trich" caused by *T. vaginalis* is reported as a highly-rated non-viral sexually transmitted disease (Malla, 2012). It primarily infects the genitourinary tracts of humans (Harp & Chowdhury, 2011; Malla, 2012). Being a widely prevalent and curable STI, human trichomoniasis has gained worldwide importance due to the significant illness and considerable economic and emotional burden that it leaves on the community (Malla et al., 2001). WHO has approximated the occurrence of trichomoniasis at a rate more than 170 million cases yearly accounting for almost half of all curable STIs globally (Schwebke, 2002; Malla, 2012) with United States reporting about eight million cases per year (Van Der Pol, 2007). Being the etiologic agent of this extremely common cosmopolitan infection in both genders, *T. vaginalis* is known as one of the widely studied parasitic protozoa worldwide (Petrin et al., 1998; Garber, 2005).

T. vaginalis is a flagellated parasite from the order *Trichomonad* and genus *Trichomonas* that potentially inhabits the genitourinary tract of the host (Schwebke & Burgess, 2004). *Trichomonads* form a large family of protozoan species with wide range of host habitat varying from humans, other primates, cattle and avians (Wang, 2000). However, only three species from the genus *Trichomonas* infects humans; *Trichomonas tenax* isolated from oral cavity, *Pentatrichomonas hominis* found in intestinal tract and *T. vaginalis* from the urogenital tract (Wang, 2000;

Schwebke & Burgess, 2004). Among these three species, only *T. vaginalis* is pathogenic and causes disease, trichomoniasis due to their unique biological and morphological properties (Petrin et al., 1998;Wang, 2000; Schwebke & Burgess, 2004). *T. vaginalis* was first identified and named by a French parasitologist known as Donné in 1936 (Thorburn, 1974) and was found to be transmitted through bodily contact (Lewis, 2005, 2010).

T. vaginalis is investigated as important human parasites as it can cause a range of infections in both males and females with predominance in latter (Valadkhani et al., 2008). Examples of infections includes urethritis (Krieger et al., 1993), vulvo-vaginitis, cervicitis (Sehgal et al., 2012), prostatitis, atypical pelvic inflammatory disease and infertility (Valadkhani et al., 2008; Noël et al., 2010). Many studies have been triggered due to:

a. increase prevalence of *T. vaginalis* infection globally (Wang, 2000).
b. the parasite being highly contagious (Wang, 2000)
c. the parasite having asymptomatic characteristics identified in some of the infected patients especially in males (Johnston & Mabey, 2008).
d. the association of trichomoniasis with other sexually transmitted diseases such as gonorrhea, cervical cancer and Human Immunodeficiency Virus (HIV) (Johnston & Mabey, 2008; Smith & Ramos, 2012).

All these factors form the basis for many research studies to be mounted on the possible mechanisms involved in phenotypic, genotypic, biochemical and biological aspects of *T. vaginalis* (Noël et al., 2010; Afzan, 2011a; Sehgal et al., 2012).

This parasite appears in a few forms i.e. trophozoites (Petrin et al., 1998) and pseudocyst (Pereira-Neves et al., 2003). These are the two notable forms that are predominantly well studied by many researchers. However, in recent years, another

morphological form of *T. vaginalis* known as the amoeboid form has been studied quite extensively. These forms exhibit properties related to adherence which contributes to the pathogenicity of the parasite (Arroyo et al., 1993; Gonzàles-Robles et al., 1995; Pereira-Neves & Benchimol, 2007).

Since the amoeboid forms are seen and that too when the parasites adhere to certain types of cells such as epithelial or vaginal cells and sometimes on the cover slips (Fiori et al., 1999), little attention was given to these forms of the parasite. Afzan & Suresh (2012a) reported that the amoeboid forms were observed on day three of the parasite culture isolated from cervical neoplasia patients. However, little is known on the factors that trigger amoeboid form to appear in cultures of *T. vaginalis* as well the transitional changes during this transformation.

1.2 Research Questions

The current study attempts to answer the following research questions:

i. Can the present study repeat the findings of Afzan and Suresh (2012a) on the formation of amoeboid forms of *T. vaginalis* on day 3 culture of cervical neoplasia patients even after prolonged maintenance in *in vitro* cultures?
ii. Do different types of stress conditions in the suspension culture trigger the formation of amoeboid forms of *T. vaginalis*?
iii. If amoeboid forms are observed, then what are the morphological properties of such forms as well as what are the transformational changes that take place to differentiate the normal flagellated trophozoites and amoeboid of *T. vaginalis*?

1.3 Justification of the study

Trichomoniasis is a urogenital disease affecting humans with higher prevalence among women than men. The urethra is the common site of infections in men while the vagina is the common site of infection in women (Lewis, 2010). Since trichomoniasis is often associated with the progression of other sexually transmitted infections, the present study was undertaken based on the findings by Yusof and Kumar (2012) who studied on parasite isolated from symptomatic women diagnosed with cervical intraepithelial neoplasia (CN) and non-cervical intraepithelial neoplasia (NCN).

Morphological characteristics of the parasite are mostly used to differentiate the pathogenic causing forms resulting from acute to severe complication in patients (Rasmussen et al., 1986; Ryan et al., 2011; Malla, 2012; Sehgal et al., 2012). The flagellated trophozoite is the most common form of *T. vaginalis* which has been well-studied by many investigators (Petrin et al., 1998; Ryu & Min, 2006; Harp & Chowdhury, 2011; Yusof & Kumar, 2012). The other form of the parasite which is the pseudocyst has recently gained wide attention following the findings by Pereira-Neves and team (2003) implicating the possible association of this form with pathogenicity of *T. vaginalis*. Abnormal shapes of *T. vaginalis,* precisely the amoeboid form has been long identified and was known to appear upon attachment with vaginal cells (Fiori et al., 1999; Tasca & De Carli, 2002). These forms have been continuously studied for their cytoadherence (Arroyo et al., 1993), cytopathogenicity (Fiori et al., 1999) and cytotoxic (Brugerolle et al., 1996) mechanisms contributing to increased risk of inflammation in women.

In 2002, Tasca and De Carli suggested to study the significance of shape variations in *T. vaginalis* as well as the influence of culture medium components on the parasite development. This was supported by the recent findings of Afzan and

Suresh (2012a) on identifying amoeboid forms in suspension culture, triggering questions on the possible factors that sustain the formation of this shape under natural conditions. However, apart from these studies, there have been no detailed investigation on the phenotypic characteristics and the factors that trigger amoeboid form of *T. vaginalis* in suspension culture isolated from cervical neoplasia patients under normal and unfavourable conditions.

Therefore, the present study was conducted to study the phenotypic characteristics of amoeboid forms of *T. vaginalis* from symptomatic cervical intraepithelial neoplasia and non-cervical intraepithelial neoplasia patients. It focused on the aspects that contribute to the appearance of amoeboid morphology by varying the culture conditions such as analyzing the effect of different concentrations of horse serum, effect of different parasite inoculum sizes and different concentrations of metronidazole. Further detection and characterization of the amoeboid forms were performed using staining method to elucidate the transformational changes from trophozhoites to amoeboid forms under light microscopy.

1.4 Objectives of study

a. To identify the phenotypic characteristics of amoeboid forms of *T. vaginalis* isolated from cervical intraepithelial neoplasia and non-cervical intraepithelial patients by means of;
    i. Generating the growth profile of amoeboid forms.
    ii. Generating the growth profile of trophozoites and correlating the numbers of trophozoite forms to the appearance of amoeboid forms.
    iii. Morphological analysis of the amoeboid forms using Giemsa and Modified Field's Stain.

b. To elucidate the factors contributing to the formation of amoeboid forms by evaluating;

   i. Effect of different inoculum sizes on the formation of amoeboid morphology.

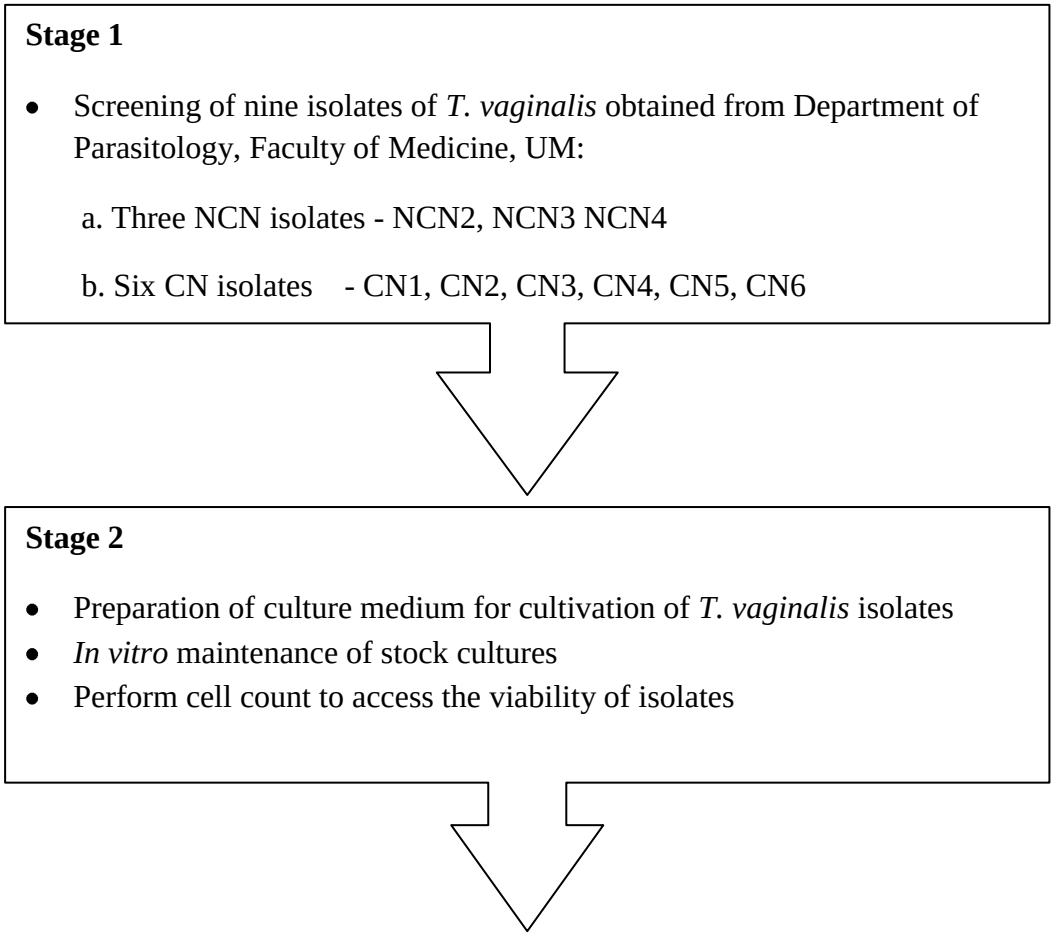
   ii. Effect of different concentration of horse serum on the formation of amoeboid morphology.

   iii. Effect of metronidazole drug on the formation of amoeboid forms.

c. To observe and compare the transformational changes of trophozoites to amoeboid forms in stressed and normal cultures using light microscopy.

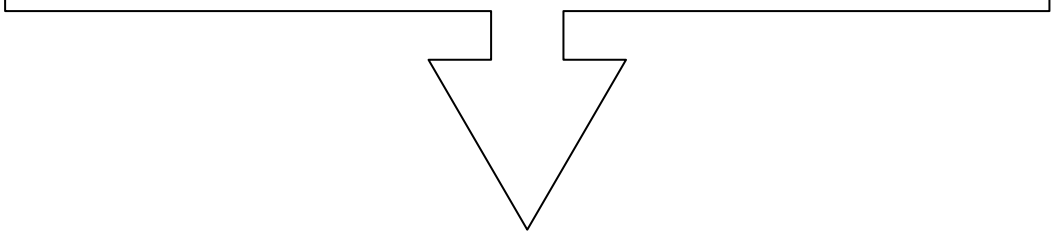
1.5 Research approach

Few approaches were undertaken in the attempt to meet the objectives of the present study encompassing four main stages as presented below:



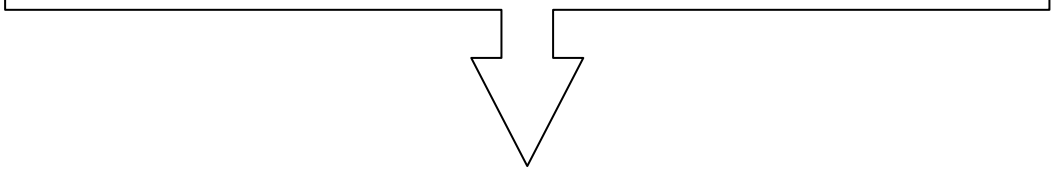
Stage 3

- Phenotypic characterization of amoeboid form of *T. vaginalis* based on:
  a. Generation of growth profiles of amoeboid forms
  b. Generation of growth profiles of trophozoites forms.
  c. Detection and morphological analysis using staining methods.
  d. Analyzing the effect of varying culturing parameters on the formation of amoeboid forms: - inoculums size, concentrations of horse serum and concentrations of metronidazole drug



Stage 4

- Further characterization of amoeboid form by observing the transformational changes from trophozoites to amoeboid under light microscopy



Stage 5

- Data analysis using SPSS to obtain data significance
- Discussion and answers to the research questions

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of *T. vaginalis*

T. vaginalis is a single-celled, flagellated protozoan parasite that was first identified and named by a French parasitologist, Alfred Francois Donné in 1836 (Thorburn, 1974; Garber, 2005). Donné observed *T. vaginalis* as a motile microorganism isolated from frothy leucorrhea of women with vaginal discharge and genital irritation (Sood & Kapil, 2008). It is a microaerotolerant protist (Johnson et al., 1993; Guschina et al., 2009; Sehgal et al., 2012) that requires human or animal host for survival and adaptation (Strous, 2008). As a primitive eukaryote, it lacks mitochondria and uses hydrogenosomes to accomplish fermentative carbohydrate metabolism (Sood & Kapil, 2008) under aerobic and anaerobic conditions(Petrin et al., 1998).

T. vaginalis is derived from the family Trichomonads; a large family of protozoan species that inhabits a variety of hosts ranging from humans and other primates to cattle and avians (Wang, 2000). Being among the three genuses of *Trichomonas* that infects human, *T. vaginalis* is the primary causative agent of the world's most common non-viral sexually transmitted disease known as trichomoniasis with an annual rate of more than 170 million cases globally (Garber, 2005; Gehrig & Efferth, 2009). Potentially, *T. vaginalis* infects the urogenital tracts in both men and women with a high dominance in the latter (Garber, 2005).

Although curable, infected patients can be asymptomatic for the infection, thus increasing the risk of being untreated (Petrin et al., 1998). Presence of trichomoniasis in asymptomatic patients has been investigated by Valadkhani and team (2008). They concluded that asymptomatic patients are healthy carriers of *T. vaginalis* and are often associated with other STDs. Predispositions to other sexual infections such as cervical

inflammation (Shafir et al., 2009) which may progress into cervical cancer (Zhang et al., 1995; Viikki et al., 2000; Pustan et al., 2010) if untreated, transmission of HIV (Moore, 2007; Van Der Pol, 2007; Shafir et al., 2009) pelvic inflammatory disease (Petrin et al., 1998) or infertility are other important characteristics of *T. vaginalis* that makes it an important parasite to be explored. Progress and challenges in the study of *T. vaginalis* has also been outlined in terms of infections in women infected with HIV, diagnosis and recurrent treatments with potential drugs (Bachmann et al., 2011).

Principal investigations on this parasite involved mainly biochemical tests and microscopic examinations to understand the growth profiles and behavior of the organisms (Sood & Kapil, 2008). Trussell and Johnson (1944) summarized few previous experimental studies relative to the parasite morphology, cultivation methods, biological properties, chemotherapy, epidemiology, pathogenicity and serology. In addition, Ovcinnikov et al. (1975) undertook an extensive study on *T. vaginalis* to identify the morphology, feeding mode mechanism, metabolism, life cycle and as well as host-parasite relationship using electron microscopy.

However, the growing infections of *T. vaginalis* has attracted many investigators to conduct extensive review and research ranging from morphological analysis up to immunological and molecular analysis to study the pathogenesis and clinical manifestations of this organism (Sood & Kapil, 2008). Klassen-Fischer and Ali (2011) have discussed trichomoniasis based on morphological description, clinical features, pathogenesis, diagnosis and treatments enhancing the understanding about this STD. Likewise, Petrin and members (1998) have given a general overview on the clinical and microbiological aspects of *T. vaginalis* through the compilation of studies from previous scientists.

2.2 Taxonomy classification

The scientific name of this parasitic protozoan is *Trichomonas vaginalis* and the diseases are known as “trich”, “vaginitis” and “ureitis” (Strous, 2008). The complex structure and the large size of *T. vaginalis* have placed it under the most primitive eukaryotic domain of Eukarya (Strous, 2008) and Kingdom Protista. This is further supported by molecular phylogenetic studies using large and small ribosomal subunits by earlier studies (Pereira-Neves et al., 2003) indicating that these organisms might be among the earliest diverging eukaryotes (Pereira-Neves et al.,2003).

Lower taxonomical classifications of *T. vaginalis* were reported from a different perspective in some studies that run parallel to recent updates on the genetic and phenotypic composition of this parasite. Being the most diverse parasitic protist of the human urogenital tracts with presence of flagella, it is stated to belong to the Phylum Zoomastiginia by Schwebke & Burgess (2004). Conversely, few other studies mention that *T. vaginalis* is well fitted into the Phylum Sarcomastigophora (Amany Mohammed, 2000; Ramos, 2005) and subphylum Zoomastigosphora or Mastigophora (Ramos, 2005; Amany, 2000) since the members of this level contains non-photosynthetic flagellates from both free-living and parasitic organisms (Ramos, 2005).

Presence of parabasal body such as Golgi associated kinetostomes, bundled microtubules and undulating membrane placed *T. vaginalis* under the Class Parabasalia (Schwebke & Burgess, 2004; Pereira-Never et al., 2003). However, review by Ramos (2005) categorized *T. vaginalis* under the Class Zoomastigophorea based on characteristics such as simple, flagellated and feeding mode by phagosytosis (Lackey, n.d.).

Almost most of the scientists agreed on the ranking of *T. vaginalis* into the Order Trichomonadida, and Family Trichomonadidae (Ramos, 2005; Schwebke and Burgess, 2004; Amany, 2000). Members of these groups consist of protozoans generally

with three to five anteriorly directed flagella and one attached to undulating membrane supported by the costa with no true cysts such as *T. vaginalis* (Amany Mohammed, 2000; Ackers, 2001; Schwebke & Burgess, 2004).

The classification of this species is continuously updated based on recent genetic findings (Goodman et al., 2011). Initially, this parasite was known to have originated from the genus *Trichomonas* by the founder of *vaginale*, Donné (1836) (Jamali et al., 2006). The name *Trichomonas vaginalis* was then based on the source of parasite from genital secretions of humans (Thorburn, 1974). However, recent findings highlighted that some strains of *T. vaginalis* might be from the genus *Trichomonasvirus* and family *Totiviridae* due to the presence of non-segmented double-stranded RNA (dsRNA) viruses in those strains.

2.3 General morphology of *T. vaginalis*

Discovery of *T. vaginalis* from the genital discharge of human by Alfred Donné in 1836 (Thorburn, 1974) became the platform for many scientists to initiate and develop boundless study on this human protozoa beginning with the elucidation of morphology followed by other in depth studies on this parasite. The first morphological identification of *T. vaginalis* as flagellated protozoan was described by Donné (1836) based on wet mount study (as cited in Jamali et al., 2006; Trussell & Johnson, 1944). When Trussell performed axenic cultivation in 1940 (Clark & Diamond, 2002), pure cultures of *T. vaginalis* were obtained easily.

Preliminary structural description of *T. vaginalis* was provided by Donné in 1837 (Afzan, 2011a) as illustrated in Figure 2.1. The parasite is known to have an axostyle, chromatic basal and granules, cytosomal fiber, karyosome, nucleus and an undulating membrane with four external flagella (Afzan, 2011a). This primary morphological description of *T. vaginalis* initiated for a more detailed structural study of the parasite by subsequent scientists along with the advancement in technology such

as by using scanning electron microscopy, transmission electron microscopy or through fluorescent staining. As an example, Costamagna and Figueroa (2001) performed an ultrastructure study on *T. vaginalis* isolated from adult women and grown in liquid medium to define the morphology of its cytoskeleton, hydrogenosomes and endocytosis phenomena.

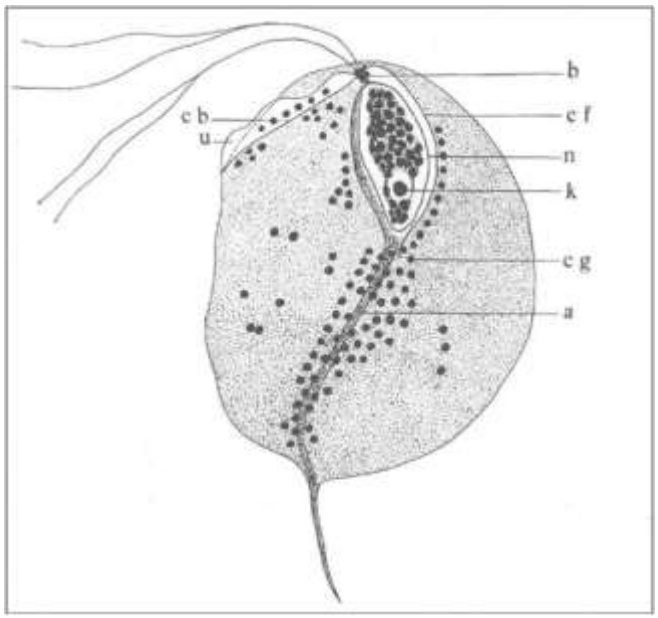


Figure 2.1: First morphological illustration of *T. vaginalis* by Donné (1837)
Note: a = axostyle; b = blepharoplastic granules; cb = chromatic basal; cf = cytosomal fiber; cg = chromatic granule; k = karyosome; n = nucleus; u = undulating membrane (Adapted from Afzan, 2011a).

T. vaginalis is typically described as free-swimming and actively motile parasite conforming to ellipsoidal, ovoidal or rounded shapes when observed under light microscopy (Petrin et al., 1998; Liang & Huang, 2009). In the absence of cell debris (Amany, 2006), *T. vaginalis* conforms to a more uniform, actively swimming pyriform or rounded shape (Honigberg & King, 1964; Ackers, 2001) with four anterior flagella at the arrangement of [9(2) + 2) and one attached to the undulating membrane (Schwebke & Burgess, 2004; Wang, 2000). Generally, *T. vaginalis* is quite robust in appearance and measures with an approximate length and width of 7-32 μm and 5-12 μm respectively (Costamagna & Figueroa, 2001; Klassen-Fischer & Ali, 2011; Afzan,

2011a). Internal organelles of *T. vaginalis* includes nucleus, axostyle and hydrogenosomes (Schwebke & Burgess, 2004), each with unique and significant functions in order to maintain the viability of the parasite. On the contrary, under unfavourable culture conditions (Petrin et al., 1998) or in the presence of site for attachment such as epithelial cells (Fiori et al., 1999), *T. vaginalis* appears abnormally different in shapes.

T. vaginalis is known to be well-accustomed to the changing vaginal environment (Bhatt et al., 1996) and thus can be highly pleiomorphic (Jesus et al., 2004) with great variation in shapes and sizes depending on the *in vitro* or *in vivo* physical and physiochemical conditions (Smith, 2012; Jesus et al., 2004; Tasca & De Carli, 2002; Petrin et al., 1998). The capability to adapt and survive in changing environmental conditions either *in vivo* or *in vitro* (Jesus et al., 2004) has demonstrated *T. vaginalis* to have various morphologies to date which includes trophozoites as a more uniform shape when swimming freely (Trussell & Johnson, 1944; Petrin et al., 1998; Ryu & Min, 2006), pseudocyst with a internalized flagella (Pereira-Neves et al., 2003; Afzan & Suresh, 2012b) and irregular amoeboid form upon contact with vaginal cells (Tasca & De Carli, 2002).

In a study by Ryu and Min (2006) on *T. vaginalis* in Republic of Korea, trophozoite forms isolated from women with vaginal trichomoniasis was clearly defined based on structural appearances such as size, shape and internal organelles using electron microscopy. Recently, Yusof and Kumar (2012) characterized trophozoite as phenotypic variant forms of *T. vaginalis* and compared the forms among cervical neoplasia isolates and non-cervical neoplasia isolates using electron microscopy and cytochemical staining methods. Meanwhile another notable form known as pseudocyst was reported by Pereira-Neves et al. (2003) and Afzan and Suresh (2012b) presenting contradictory data to previous study (Petrin et al., 1998) that reported formation of

pseudocysts under degenerative and unfavourable conditions. Significantly, another form of *T. vaginalis* known as amoeboid forms were also identified as an integral shape of the parasite and has been reported by few authors (John & Squires, 1978a; Arroyo et al., 1993; Jesus et al., 2004) based on their morphological appearance, pathogenicity and adherence mechanism.

Studies have also suggested that virulence of *T. vaginalis* strains may play a role in the different morphologies of the parasite (Tasca & De Carli, 2002; Jesus et al., 2004; Adegbaju & Morenikeji, 2008). Apart from trophozoite, the other morphological forms that appear without flagella were assumed as part of developmental process of the parasite instead of being different stages in the life cycle of *T. vaginalis* (Malla et al., 2001).

2.3.1 Trophozoite form

Trophozoites form is the initial and dominant shape identified in developing stages of *T. vaginalis* that has been structurally studied in detail using scanning electron microscopy and transmission electron microscopy by many investigators. Trophozoite of *T. vaginalis* was observed as free-swimming (Afzan, 2011a; Honigberg & King, 1964), ovoid or pyriform in shape (Klassen-Fischer & Ali, 2011; Costamagna & Figueroa, 2001) with approximate length and width ranging within 7-30 µm and 5-15 µm respectively (Ryu &Min, 2006; Costamagna & Figueroa, 2001). The overall structural appearance of *T. vaginalis* trophozoite is illustrated in Figure 2.2.

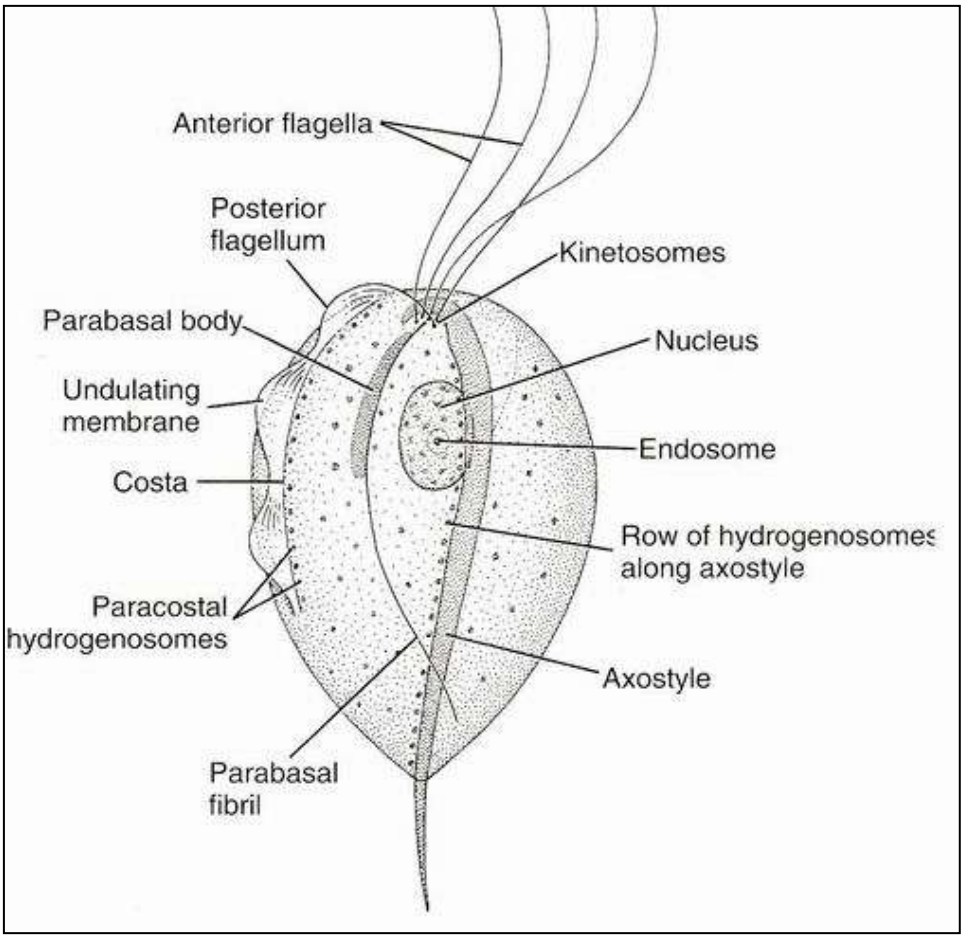


Figure 2.2: Trophozoite form of *T. vaginalis*. (Adapted from Strous, 2008).

The trophozoite consists of five flagella contributing to its motility; four at the anterior portion and one incorporated within the undulating membrane at the posterior portion of the parasite (Petrin et al., 1998; Ryu & Min, 2006; Liang & Huang, 2009). An axostyle, a rigid structure located in the center of the tropozhoite holds part of the barbwire-like posterior flagellum (Strous, 2008; Smith, 2012). The axostyle is a slender hyaline, rod-like structure known to divide the protozoa longitudinally and protrudes through the posterior end to form a sharp point (Petrin et al., 1998; Adegbaju & Morenikeji, 2008) enabling the parasite to attach to vaginal epithelial cells (Petrin et al., 1998).

A large single and round nucleus surrounded by porous nuclear envelope is positioned at the anterior part (Adegbaju & Morenikeji, 2008) of *T. vaginalis* along with prominent Golgi complex known as parabasal body attached by fibrils to the kinestome of the flagella (Klassen-Fischer & Ali, 2011; Petrin et al., 1998). The parasite is evenly distributed with nuclear chromatin and hydrogenosomes are found evidently along the axostyle and costa which supports the undulating membrane (Petrin et al., 1998; Ryu & Min, 2006; Adegbaju & Morenikeji, 2008). The hydrogenosomes are formerly known as siderophil granules and serve as mitochondria since trophozoite of *T. vaginalis* does not possess the latter in the cytoplasm (Ryu & Min, 2006; Petrin et al., 1998). Named after the ability to produce molecular hydrogen as end product of metabolism, hydrogenosomes plays an important role in energy production and drug activation to support the metabolic activity of *T. vaginalis* (Ryu & Min, 2006). Morphological description of trophozoite form of *T. vaginalis* has begun in late 1930s by Powell following the identification of the parasite by Donné in 1836. This was then further discussed by Trussell and Johnson (1944) in their review on experimental studies based on morphology, pure culture study, biology, chemotherapy, epidemiology, pathogenicity and serology of this protozoa. Further studies of *T. vaginalis* for fine and detail surface structure analysis were performed by Ovcinnikov and team (1975) using transmission and scanning electron microscopy. Recently, Ryu and Min (2006) reported the occurrence of only trophozoite stage in *T. vaginalis* from vaginal samples of women in Republic of Korea and detailed its structural components using electron microscope. In another study, Yusof and Kumar, 2012 demonstrated trophozoite as phenotypic "variant" forms of *T. vaginalis* by comparing its biological, biochemical and ultrastructure characteristics with isolates from cervical neoplasia and non-cervical neoplasia in Malaysia.

2.3.2 Pseudocysts form

Another significant morphology of *T. vaginalis* that deforms the trophozoites under certain conditions is pseudocyst as proved by Pereira-Neves et al. (2003). Unlike trophozoite, the pseudocyst is defined as a compact, non-motile, without a true cyst wall, round in shape and lack of external flagella (Figure 2.3) (Pereira-Neves et al., 2003). Initially, pseudocyst was reported as degenerative forms of *T. vaginalis* that appeared when the culture conditions were unfavourable (Petrin et al., 1998) and its formation remains uncertain.

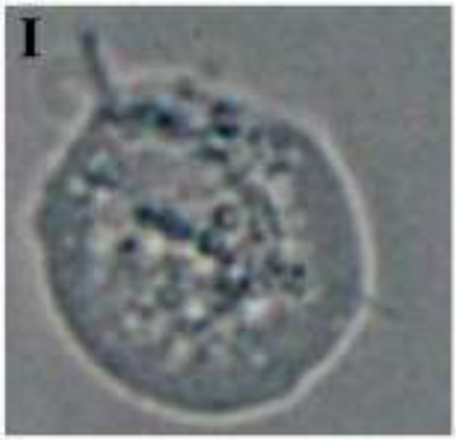


Figure 2.3: Epifluorescence image of pseudosyst of *T. vaginalis* stained with Acridine orange. Magnifications 400X (Yusof and Kumar, 2012).

However, recent findings proved that pseudocyst plays a significant role in triggering trichomoniasis (Hussein & Atwa, 2008) and can be reversed to trophozhoites under certain circumstances (Afzan & Suresh, 2012b; Pereira-Neves et al., 2003). According to Pereira-Neves et al. (2003), pseudocyst can appear under natural conditions and also when induced using hydroxyurea methods. The study analysed the structural changes of pseudocyst using immunofluorescence and video microscopy. The team concluded that pseudocysts differs from trophozoites and are competent to divide

suggesting that it may play another role in sustaining trichomoniasis. Similarly, study on mice injected intra-vaginally with *T. vaginalis* pseudocysts by Hussein and Atwa (2008) proved the occurrence of trichomoniasis in the infected mice and hence supporting the active contribution of pseudocsyts in causing the disease.

2.3.3 Amoeboid form

Amoeboid form of *T. vaginalis* is another distinctive morphology of this mucosal protozoan that is currently being studied extensively. This form (Fig. 2.4) is known to be flattened, non-motile or slowly motile with an inactive flagella and a delicate outer layer projecting pseudopodia-like cytoplasmic extensions (Heath, 1981; Fiori, et al., 1999; Tasca & De Carli, 2002).

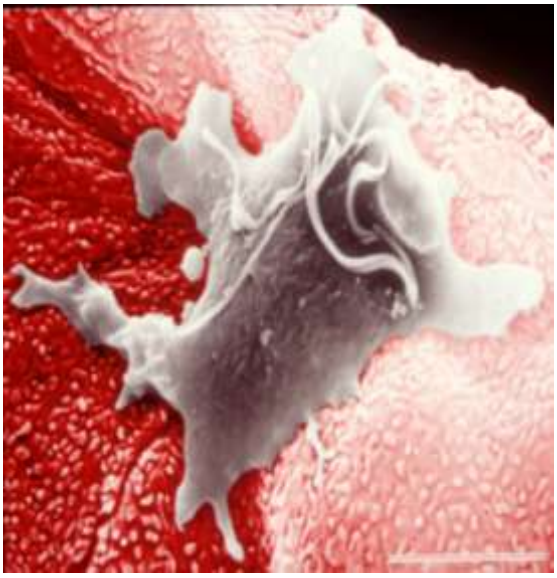


Figure 2.4: Electron micrograph of amoeboid form of *T. vaginalis*. (Alderete, 2012).

Amoeboid shape were observed only when the parasites adheres to the vaginal epithelial cells (Arroyo et al., 1993; Petrin et al., 1998; Fiori et al.,1999), attaches to erythrocytes (Tasca & De Carli, 2002) or onto glass cover slips (Brugerolle et al., 1996). However, a very recent and significant finding by Afzan and Suresh (2012a) found that amoeboid form of *T. vaginalis* can also appear naturally in axenic culture medium

without any attachments and was proved with growth profile study and ultrastructure analysis with electron microscopes.

The primary factor that triggers the appearance of amoeboid form of *T. vaginalis* apart from trophozoite and pseudocysts is the ability of the parasite to conform adherence to the vaginal epithelial cells when opportunity arises, also known as host-parasite relationship in order to maintain survivability of this extracellular parasite (Ryan et al., 2011). In conjunction to the adhesion properties of *T. vaginalis* that completely transforms the free-swimming trophozoites into immobile amoeboid, morphological description of this parasite takes a new deviation with focusing on the mechanisms involved in triggering this unusual shape (Fiori et al., 1999). This adherence property of *T. vaginalis* also makes amoeboid strains to be the most virulent as elucidated by Brugerolle et al. (1996) with the presence of actin cytoskeleton in *T. vaginalis* compared to other trichomonads.

Although abnormal shapes of *T. vaginalis* was reported as early as 1978 by John and Squires, however only recently the relation between the infective mechanisms of the parasite that destroys host cells was elucidated with great emphasis on cytotoxicity and cytopathogenicity (Rasmussen et al., 1986; Arroyo et al., 1998; Fiori et al., 1999; Adegbaju & Morenikeji, 2008; Alderete, 2012). Despite the findings that only trophozoite causes trichomoniasis, a biochemical analysis of amoeboid form conducted by Scott et al. (1995) concluded that amoeboid and flagellated form of *T. vaginalis* synthesizes similar proteinases that is also responsible in causing trichomoniasis in human. In a study conducted by Tasca and De Carli (2002), amoeboid form has been observed as abnormal form of *T. vaginalis* using scanning electron microscopy that appeared prior inoculation with McCoy cell monolayers and human erythrocytes. The amoeboid form was reported to have pseudopodia-like extensions used for feeding and

attachment to stationary objects but not for amoeboid movement (Tasca & De Carli, 2002).

Virulence of the *T. vaginalis* strain also plays an important role in triggering the amoeboid formation due to the changes that it causes to the microenvironment of the growth culture enhancing the secretion of adhesins protein responsible for the attachment properties of the parasite (Arroyo et al., 1998; Fiori et al., 1999; Liang &Huang, 2009). As reported by Jesus et al. (2004), freshly isolated strains of *T. vaginalis* presented higher percentage of amoeboid form with more virulence characteristics based on cytolytic activity compared to prolonged sub-cultured strains. The appearance of morphological variability among the strains highlighting the amoeboid form were determined using different environmental effects such as temperature, pH, oxygen tension, carbohydrates and contact with other cell types (Jesus et al., 2004). Likewise, Figueroa-Angulo and team (2012) revealed the effects of environmental factors on the virulence of *T. vaginalis*. They deduced amoeboid form as the key player of pathogenesis based on morphological transition observed under scanning electron microscope when the parasite attaches to cervical and prostatic cells.

2.4 Reproduction and life cycle of *T. vaginalis*

It is significant to understand the reproduction mechanism and life cycle of *T. vaginalis* in order to relate the formation of different morphology within this parasite as well as to understand the pathway of trichomoniasis infections. In the case of *T. vaginalis,* the life cycle is still poorly understood (Sood & Kapil, 2008; Petrin et al., 1998) and is persistently being studied.

The life cycle of this human pathogenic protozoan is simple (Schwebke & Burgess, 2004) such that it comprises only the infective trophozoite form and lacks the cyst stage (Strous, 2008; CDC, 2009). The overall diagrammatic overview on the reproduction stage of *T. vaginalis* adapted from CDC (2009) is shown in Figure 2.5.

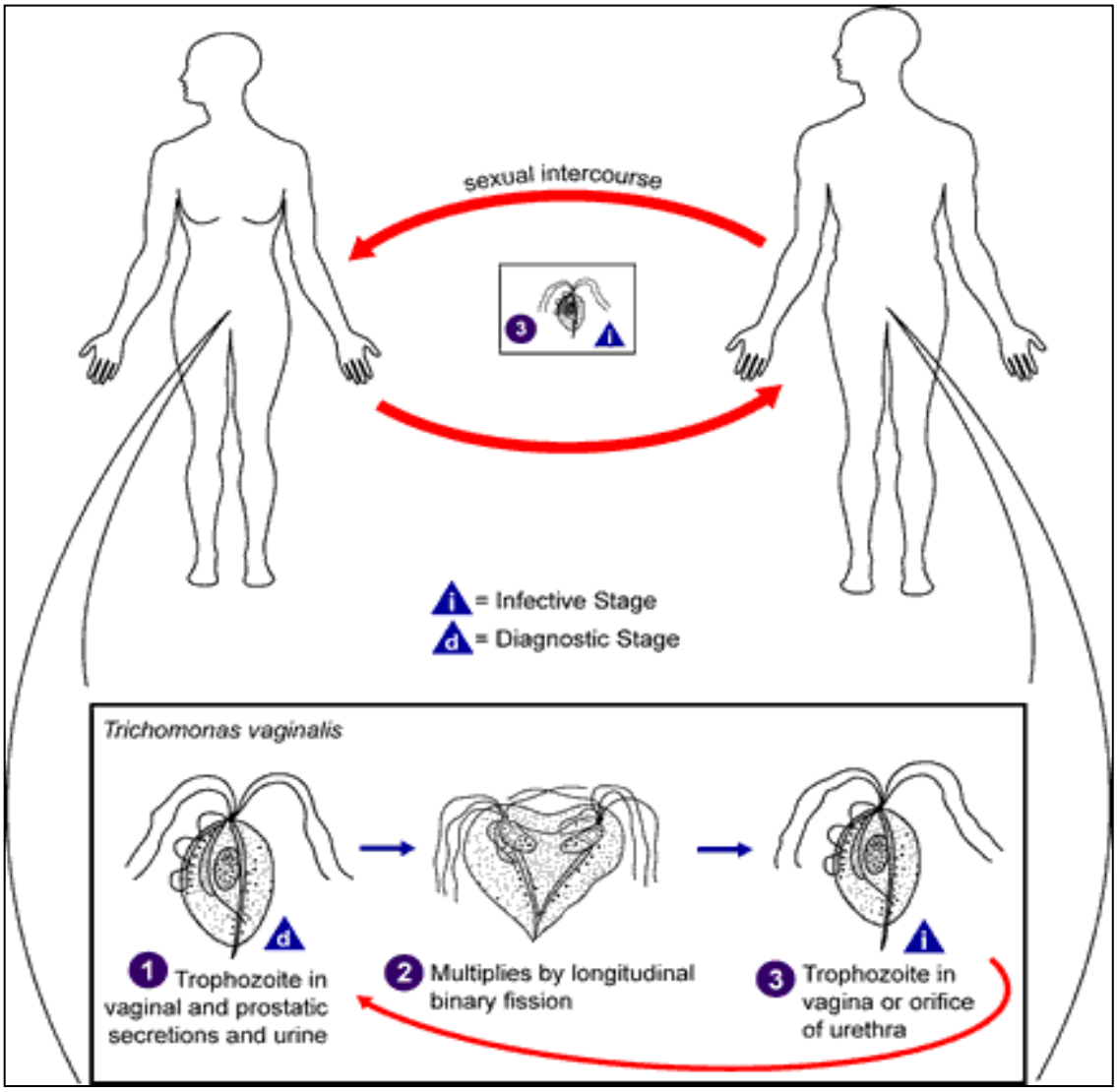


Figure 2.5: The life cycle of *T. vaginalis* (Adapted from CDC, 2009).

Trophozoites of *T. vaginalis* inhabits the female lower genital tract and the male urethra and prostate ❶, where it replicates by binary fission ❷. The parasite does not appear to have a cyst form, and does not survive well in the external environment. *T. vaginalis* is transmitted among humans primarily by sexual intercourse ❸ (CDC, 2009).

The flagellated trophozoite of *T. vaginalis* is known to reproduce asexually (Afzan and Kumar, 2011a) primarily by longitudinal binary fission and under natural infections, the parasite expand the population in the lumen and mucosal surfaces of the urogenital tracts of human (Sehgal et al., 2012; Schwebke & Burgess, 2004). During reproduction, the nuclear membrane surrounding the nucleus of *T. vaginalis* is retained throughout the dividing period giving rise to infective progeny (Adegbaju & Morenikeji, 2008). Petrin and team (1998) explained that apart from trophozoite, several

other oversized round forms of the trichomonad known to exist in dividing growth phase culture are not stages in the life cycle. Instead the shape was triggered due to certain unfavourable conditions.

However, another mode of reproduction that has been identified recently is the multiple nuclear fission that gave rise to abnormal forms of *T. vaginalis* under favourable conditions (John & Squires, 1978a; Yusof and Kumar, 2011). One such example is proved by findings of Yusof and Kumar (2011) that observed multi-nucleated *T. vaginalis* using cytochemical staining method and ultrastructural analysis to visualize the events taking place during the transformation of single nucleus to multinucleated *T. vaginalis* which eventually turned the ovoid trophozoite to an abnormally large and irregularly shaped amoeboid.

2.5 Mode of transmission

Humans are the only natural host for *T. vaginalis* (Petrin et al., 1998). Since trichomoniasis is classified as a sexually transmitted disease, thus it is clear that the common mode of transmission is through sexual intercourse (Petrin et al., 1998; Coleman et al., 2013) between sexually different partners or within the same sexual partners (Coleman et al., 2013) with either one or both being infected. Moore (2007) reported that male-to-female transmission rates were higher than female-to-male rates with approximately 85% of exposed women were said to contract the disease. Moreover, prevalence of trichomoniasis was reported to be higher among women with multiple sexual partners and in women with other sexually transmitted infections (Soper, 2004). In a study conducted in Iran, trichomoniasis was proved to be prevalent among women involved actively in sexual activities compared to those of under-age or nearing menopause (Chalechale & Karimi, 2010) suggesting that sexual intercourse is the main cause of the disease.

Transmission is said to occur through the infective trophozoite stage found in the vaginal or urethral discharges of infected persons ("*Trichomonas vaginalis*," 2010). Trophozoite requires body fluids and moist surfaces for survival (Lewis, 2010) and dies at dried conditions (Ryu & Min, 2006). Once the trophozoites are transmitted through the urogenital tract of another individual, it then colonizes the epithelial surface of the tract to obtain nutrients and expand the growth through multiplication (Sehgal et al., 2012) which later develops into infection. The incubation period of *T. vaginalis* before initiating an infection is between 4 to 28 days (Coleman et al., 2013; Klassen-Fischer & Ali, 2011; Moore, 2007).

Conversely, non-sexual transmission of *T. vaginalis* has also been reported although this type of transmission is uncommon and occurs at rare circumstances ("*Trichomonas vaginalis*", 2010). Crucitti and team (2011) postulated incidence of trichomoniasis among virgin girls in Zambia due to sharing of bathing water and inconsistent use of soap. Survivability of *T. vaginalis* besides in the body fluids of human was also proved by Pereira-Neves and Benchimol (2008) through the supportive data showing the viability and cytotoxicity of the strains grown in swimming pool indicating the possibility of acquiring trichomoniasis through non-sexual mode. Although the infection rate through non-sexual mode is low (Ryu & Min, 2006), yet transmission through formites such as contaminated douch nozzles, moist wash-clothes, specula or toilet seats ("*Trichomonas vaginalis*", 2010; Moore, 2007; Petrin et al., 1998) were still possible if the parasite successfully find their way into the vagina. Transmission via non-sexual contact is possible due to the fact that *T. vaginalis* are able to survive persistently for a long time outside the body if the external environment is sufficiently moist (Ackers, 2001).

Another probable mode of transmission identified was through vertical vaginal delivery (Coleman et al., 2013) when newborn acquires infection through infected

mother during delivery (Lewis, 2010; Petrin et al., 1998). Schwandt and team (2008) reported the occurrence of trichomoniasis in a newborn on day 19 after birth was hypothesized as perinatal transmission. Petrin and members (1998) also indicated that transmission to newborn infants occurred in 2 % to 17 % of cases resulting in urinary or vaginal infections.

2.6 Etiology

Main etiologic agent of trichomoniasis is a parasitic protozoan known as *T. vaginalis* (Schwebke & Burgess, 2004; Petrin et al., 1998). Extensive studies have proved that *T. vaginalis* is the primary cause of trichomoniasis in women, however only few were conducted in men. One of the recent study confirmed the significant role of *T. vaginalis* and *Mycoplasma genitalium* as the main etiological agent of urethral discharge syndrome in West African men (Pépin et al., 2001).

Risk factors associated with the occurrence of trichomoniasis due to sexual activity includes non-use of condoms or oral contraceptives, low socioeconomic classes (Lewis, 2010), use of injection drugs, constant change of sexual partners, history of STIs, and exchanging sex for drugs or money (Smith & Ramos, 2012). Based on the risk factors analysis carried out by Miller et al. (2008), it was proved that usage of drug for a past 30 days resulted in most cases of trichomoniasis among African-American women in New York City compared to having multiple male partners. A prospective study in China by Zhang (1996) were able to prove the relationship between *T. vaginalis* and life style of women such as smoking, drinking alcohol and personal hygiene as associated risk factors of trichomoniasis with women indulged in multiple sexual partner reporting as high-risk populations of trichomoniasis.

2.7 Epidemiology

In recent years, human trichomoniasis prominently emerged as the most prevalent non-viral sexually transmitted disease of parasitic origin (Malla et al., 2001) with more than 170 million cases reported annually worldwide (Swygard et al., 2003; WHO, 2005; Smith & Ramos, 2012) of which 154 million cases occurs in resource-limited areas (Johnston & Mabey, 2008). The estimations of global prevalence by WHO (2005) were made based on assumptions of wet mount sensitivity ranging from 60 % to 80 % (Van Der Pol, 2007) with recent update suggesting the sensitivity using Polymerase Chain Reaction (PCR) maybe lower, ranging from 35 % to 60 %, thus underestimating the global prevalence data (Johnston & Mabey, 2008; Malla, 2012).

Trichomoniasis has been reported with broad-based distribution in almost every region and climate and without seasonal variability regardless of races and socioeconomic levels (Sood & Kapil, 2008; Petrin et al., 1998). Although trichomoniasis is considered as most widespread STD, however it receives the least public attention (Van Der Pol, 2007) and is a non-reportable disease (Schwebke & Burgess, 2004) due to the facts that the infections accounts for almost half of all curable infections worldwide as presented by WHO (Van Der Pol, 2007; Schwebke & Burgess, 2004).

Prior to the global prevalence report presented by WHO (2005), one third of cases in females are thought to be asymptomatic with majority developing symptoms and signs of vaginal discharge, vulval irritation and inflammation (Johnston & Mabey, 2008). Incidence in adolescent women were reported to be high with untreated infections remained undetected for three months or longer classifying them as high risk groups for STIs (Van Der Pol et al., 2005). Meanwhile, *T. vaginalis* infections in men tend to be less apparent (Smith & Ramos, 2012) with majority being asymptomatic (Johnston & Mabey, 2008). Approximately 90 % of men in a report were shown to be

asymptomatic (Wang, 2000) and often considered to be asymptomatic carriers but playing an important role in transmitting the disease (Soper, 2004). Another report presented that the rate of transmission from men to women were higher, approximately 67 to 100% than women to men, approximately 14 % to 80 % suggesting the self-limiting characteristic of the disease in men (Malla, 2012).

Various investigators conducted different types of epidemiological studies based on geographical locations, age, races, socioeconomic status, sexual activity, number of sexual partners, other STDs, sexual customs, phase of menstrual cycle, techniques of examination (Klassen-Fischer & Ali, 2011) specimen collection and laboratory procedures (Petrin et al., 1998). A study by Sutton et al. (2007) revealed the prevalence of *T. vaginalis* infection among non-Hispanic black women in United States was 10.3 times higher than that among non-Hispanic white and Mexican American women. Similarly, high prevalence of *T. vaginalis* infection was reported among socially-marginalized young women from low income communities in coastal Peru with incidence rate of 9.1 % (Leon et al., 2009). Survey among pregnant women revealed that 12.6 % out of 13816 women from six urban clinic centers were colonized with trichomoniasis with majority having bad behavioral activities (Cotch et al., 1991).

In normal populations, cases of trichomoniasis were approximated at about 10 % though it varies between 0 % to 65 % in different geographical locations (Malla, 2001), age groups and population studied (Malla, 2012). Annually in United States, trichomoniasis is identified as an extremely common cases (Schwebke & Burgess, 2004) estimated at a rate of 8 million cases (Malla, 2012) with five million new cases among women exceeding the cases of other types of STIs such as chlamydia and gonococcal (Moore, 2007). A review by Petrin et al. (1998) stated the prevalence of vaginal trichomoniasis among African populations between 11 and 25 % meanwhile an increasing rate of prevalence (approximately 10.4 %) have been reported among women

in Republic Korea since 1947 till recently (Ryu & Min, 2006). In United Kingdom, about 93 % women and 7 % men were infected with trichomoniasis (Lewis, 2010) meanwhile prevalence rate ranges between 15 to 20 % among tropical populations in Asia (Afzan, 2011a). In males attending STD clinics, *T. vaginalis* was reported as common especially in those 30 years or older accounting for as much as urethritis such as gonoccocal and chylamdia infections (Joyner et al., 2000).

Despite the significantly high prevalence rates worldwide, cases of trichomoniasis reported were relatively low in Malaysia with most cases among women who attended local clinics for inspections (Amal et al., 2010). According to this report, no cases of trichomoniasis were reported in STD clinics and only 0.36 % was detected positive in Lembaga Penduduk dan Pembangunan Keluarga Negara (LPPKN) clinics. The study postulated that the low prevalence rates among Chinese women could be attributed to the use of herbal and traditional medicine washes for cleaning external and internal vaginal areas. Similarly, in the study undertaken by Afzan (2011c) in 695 women who attended a local gynecology clinic, only 1.58 % of the patients were tested positive for *T. vaginalis* with 0.86 % diagnosed with cervical intraepithelial neoplasia.

2.8 Clinical manifestation of *T. vaginalis*

Trichomoniasis presents a wide variety of clinical patterns both in males and females (Petrin et al., 1998). *T. vaginalis* is a well-known urogenital parasite that infects the squamous epithelium in the genital tract (Petrin et al., 1998). Once established, the infection persists for a long duration in females but only lasts for a short period in males (Petrin et al., 1998).

2.8.1 Clinical manifestation in women

The spectrum of trichomoniasis in women ranges from asymptomatic carrier state (Malla et al., 2001; Petrin et al., 1998) to flagrant vaginitis (Petrin et al., 1998), with one third of asymptomatic patients being symptomatic within six months of infection (Malla et al., 2001; Petrin et al., 1998). The parasite is normally isolated from the vaginal area with of urethra and Skene's gland being the common infection site (Lewis, 2005, 2010). It is still unclear on the factors that influence the host inflammatory response to *T. vaginalis,* however probable causes identified includes hormonal levels, the coexisting vaginal flora, the strain and relative concentration of the organisms present in the vagina (Schwebke & Burgess, 2004). Severity of trichomoniasis infections in females are classified as acute, chronic or asymptomatic (Petrin et al., 1998).

Females with acute infection experiences diffuse vulvitis due to copious leucorrhea (Petrin et al., 1998) with malodorous, frothy, yellow or green and mucopurulent discharge (Petrin et al., 1998). The discharge is typically pooled in the posterior vaginal formix with bubble formation around 10 to 33 percent as observed through perspeculum examination (Malla, 2012). These bubbles are characteristics of the infection which appears as a result of carbohydrate metabolism which produces carbon dioxide and hydrogen gases (Moore, 2007). Likewise, small punctate hemorrhagic spots also known as "strawberry appearance" (Figure 2.6) were found on the vaginal and cervical mucosa in two percent of patients (Malla, 2012; Petrin et al., 1998). These signs and symptoms in women infected with acute trichomoniasis are rotational and worsen during menstruation period (Petrin et al., 1998). A study by Dan and Sobel (1996) determined the presence of florid inflammation with high numbers of polymorphonuclear leukocytes and parasitic load in symptomatic women who attended a chronic vaginitis clinic. A prevalence study in Nigerian women also identified the

common features of vaginal discharge in *T. vaginalis* as 69.8 % being white, 20.1 % as yellow and 28.2 % as clear with heavy and malodorous leakage (Anorlu et al., 2001).

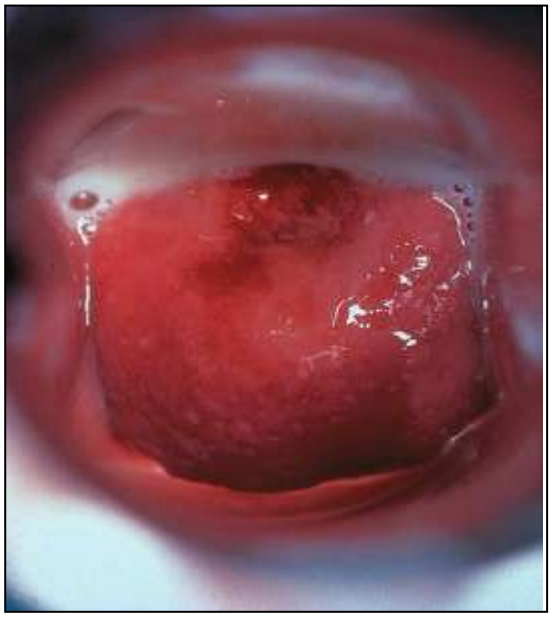


Figure 2.6: Frothy vaginal discharge of trichomoniasis with strawberry spots. (Adapted from Lewis, 2010).

Females with chronic infections were the major source of the parasite transmission with major mild symptoms and scanty vaginal secretion (Petrin et al., 1998). Complains of vaginal discharge (50 % to 75 %), pruritus (25 % to 50 %), dysuria (30 % to 50 %), dyspareunia (10 % to 50 %) and lower abdominal pain in small percentage were very common among infected females with chronic onset (Malla, 2012; Malla et al., 2001). Occurrence of dysuria and vaginal discharge were caused by a urethral infection whereas dyspareunia generally resulted from the inflamed and friable cervix (Moore, 2007).

Majority of women, around 25 to 50 % display asymptomatic state of infection with normal vaginal pH of 3.8 to 4.2 and normal vaginal flora (Petrin et al., 1998). This figure increased greatly during pregnancy while menstruation also was thought to worsen the symptoms in infected women (Malla et al., 2001). Infection during pregnancy can also be associated with premature rupture of membrane, preterm

delivery, low birth weight babies and as well respiratory infection in infants (Malla, 2012). Cotch et al. (1997) confirmed the relationship of *T. vaginalis* with adverse outcome of pregnancy by providing statistical significance on the number of low birth weight infant, preterm delivery and preterm low birth weight infant in pregnant infected women.

Complications such as adnexitis, pyosalpinx, endometritis, infertility, cervical erosion (Petrin et al., 1998) with predisposition to malignant transformation (Malla et al., 2001) were also associated with trichomoniasis. A study between 240 infertile women and 40 pregnant women revealed a clinically significant data on the role of *T. vaginalis* in causing female infertility (El-Shazly et al., 2001). Increased risk to acquisition of HIV infection was also possible with the presence of genital inflammation in infected women (Malla et al., 2001; Petrin et al., 1998). In all cases, trichomoniasis in women is suggested to continue causing infection for three to five years if untreated earlier (Swygard et al., 2003).

2.8.2 Clinical manifestation in men

Although trichomoniasis is a dominant disease in women, however infection in males still persist but in less probability, approximately five percent (Swygard et al., 2003; Petrin et al., 1998) with 70 % (Harp & Chowdhury, 2011) of them being asymptomatic carrier (Petrin et al., 1998). Symptomatic men usually exhibit non-specific urethritis (Lewis, 2010) with slight scanty, clear to mucopurulent discharge, irritation inside the penis and mild burning sensation immediately after sexual intercourse or urination (Petrin et al., 1998). Severe symptoms were also reported in cases with infections that involves the prostate or seminal vesicles (Moore, 2007). Besides, men with *T. vaginalis* as a cause of nongonococcocal urethritis also experienced symptoms for almost twice longer than men with other causative agents (Moore, 2007). However, the amount of discharge in men were usually far less then in

women as mentioned by Rein and Queiroz (as cited in Afzan, 2011a). The duration of infection is 10 days or less (Petrin et al., 1998) suggesting the less frequency of this disease in males than in females.

T. vaginalis mainly infects the urethra, epididymis and prostate gland in men (Lewis, 2010). At rare circumstances, the parasite causes clinically evident syndromes such as balanitis, epididymitis, prostatitis (Lewis, 2010), urethral disease, infertility (Petrin et al., 1998) and epidymo-orchitis (Harp & Chowdhury, 2011). Other types of complications in men associated with *T. vaginalis* infections include HIV transmission and infectivity (Soper, 2004), impaired sperm mobility and reduced sperm viability (Lewis, 2005).

Urogenital trichomoniasis in men are classified into three categories namely asymptomatic carrier state caused by sexual contact with infected women, acute trichomoniasis identified by profuse purulent nongonococcal urethritis and mild symptomatic disease (Petrin et al., 1998). A high prevalence of urethritis was found in rural men in Tanzania according to a community based study conducted with majority of them being asymptomatic for the infections (Watson-Jones et al., 2000). Similarly, another prospective study of *T. vaginalis* infection conducted in male sexual partners found that majority of them acquired the disease from their infected women partner (Seña et al., 2007). The study also revealed that 76.8 % out of 177 men examined were asymptomatic for the disease with minority of them having other symptoms such as penile discharge, penile tingling and painful urination.

2.9 Pathogenesis

Despite the mild to severe infections imposed by *T. vaginalis* causing different kinds of clinical signs and symptoms in both men and women, yet the actual pathogenesis mechanism underlying this disease has not been clearly explained (Petrin et al., 1998). The insufficient evidence to reveal the pathogenecity of *T. vaginalis*

infection is probably due to lack of good animal model in order to perform a standardized and controlled research on transmission, pathogenesis, immunity and vaccine development related to trichomoniasis (Adegbaju & Morenikeji, 2008). It was known that *T. vaginalis* damages the host tissue by direct contact and by cytotoxicity through proteins found on this parasite (Klassen-Fischer & Ali, 2011).

Different clinical spectrums identified in males and females were reported to be contributed by the multi-faceted interplay of virulence factors between parasite and host (Malla, 2012; Malla et al., 2001). However, the relationship between the putative virulence factors and the observed signs and symptoms remains unclear (Ackers, 2001). Infected men usually serve as vector to spread the disease such as urethritis and prostatitis as well as act as a co-factor to increase predisposition to HIV infection (Afzan, 2011; Soper, 2004) by disrupting the prostate epithelial cells (Afzan, 2011). Conversely, Cook (2009) mentioned that in women, *T. vaginalis* preferably attaches to squamous epithelial cells without invading the tissue in order to cause the disease (as cited in Afzan, 2011a).

Many strains of *T. vaginalis* were widely recognized as inherently pathogenic and can potentially cause serious illness to human (Heath, 1981). In addition, the host-parasite relationship is very complex (Sood & Kapil, 2008; Petrin et al., 1998) and single pathogenic mechanisms is unlikely to cause the broad range of clinical symptoms in male and female (Sood & Kapil, 2008). Cytopathogenecity of *T. vaginalis* has been well proved by Gilbert and team (2000) through the cytotoxicity tests conducted in which the human vaginal epithelial cells were destroyed as results of infection with the parasite. The study also hypothesized the significance of host-parasite relationship for the pathogenesis to occur. Following that, initial events leading to successful invasion of *T. vaginalis* have been well linked to the existence of several virulence mechanisms (Figueroa-Angulo et al., 2012) such as cell-to-cell adhesions (Lewis, 2010; Adegbaju &

Morenikeji, 2008; Petrin et al., 1998), hemolytic activity (Malla, 2012; Malla et al., 2001; Petrin et al., 1998), trichomonad proteinase activity (Ryu & Min, 2006; Petrin et al., 1998), contact-independent mechanisms and cell-detaching factor (Sood & Kapil, 2008; Petrin et al., 1998) phagocytosis (Figueroa-Angulo et al., 2012; Harp & Chowdhury, 2011) and host immune system evasion (Harp & Chowdhury, 2011; Ryu & Min, 2006; Petrin et al., 1998).

2.9.1 Cell-to-cell adhesion mechanisms

One of the initial steps in the infection process of *T. vaginalis* is through adherence mechanisms by colonization of the vaginal tract (Harp & Chowdhury, 2011). Protein components found on the cell surfaces of trichomonads is essential for adhesion, host-parasite interaction, nutrient absorption as well as exhibiting virulence property (Petrin et al., 1998). As an extracellular parasite, *T. vaginalis* needs to adhere to the epithelial cells of the urogenital tract for survival purposes (Ryan et al., 2011). Hence, adherence molecules are fundamentally important in exerting trichomonal infection (Moreno-Brito et al., 2005). Four types of adhesion proteins is known to be responsible in triggering the adhesion of the parasite to the epithelial cells; AP65, AP51, AP33 and AP23 (Alderete & Garza, 1985; Petrin, et al., 1998).

Upon attachment, the ovoid trophozoite transforms into amoeboid form that can be highly adherent (Ryan et al., 2011). Arroyo and team (1993) has clearly demonstrated the rapid amoeboid transformation of *T. vaginalis* during cytoadherence activity. The formation of cytoplasmic-like projections through the amoeboid form increases the cell-to-cell surface contact (Harp & Chowdhury, 2011) and simultaneously destroys the normal host cell, causing it to be infected. The morphological transformation leads to tight association between the parasite and host cell, thus isolating the space between membrane and the environment (Gonzàles-Robles et al., 1995). Expression of several genes in the target cells was known as the key factors to

initiate the adherence mechanism of the parasite. This was hypothesized by a study that observed the gene expression in human vaginal epithelial cells due to complex signaling cascade in response to trichomonal adherence (Kucknoor et al., 2005).

2.9.2 Hemolytic activity

T. vaginalis lacks the ability to synthesize many pyrimidines and lipids required for a more stable infection (Adegbaju & Morenikeji, 2008; Petrin et al., 1998). In return, these nutrients are acquired from erythrocytes found in the host cells which are rich in fatty acids (Petrin et al., 1998). Iron on the other hand is an essential nutrient required by *T. vaginalis* for growth in high concentration (Sehgal et al., 2012). In order to achieve this, *T. vaginalis* lyses the erythrocytes via protein receptors found on the surfaces of both the red blood cells and the parasites (Petrin et al., 1998). This hemolytic activity is said to be correlated to the virulence of the parasite (Sood & Kapil, 2008; Petrin et al., 1998). Study by Rosset et al. (2002) revealed the hemolytic activity of *T. vaginalis* as an important mechanisms in causing injury to the erythrocytes of the host cell. Electron microscopy studies (Gonzàles- Robles et al., 1995; Arroyo et al., 1993) have shown that during infection, there is a close association between the parasite and target cell whereby amoeboid morphology take control. This induces the balancing of the microenvironment by the parasite by means of increasing the concentration of pore-forming proteins needed to achieve lysis activity.

2.9.3 Proteinase activity

Trichomonad proteinases have been related to various types of pathogenesis which includes nutrients absorption, immune system invasion, cytotoxicity, cytoadherence and hemolysis (Alvarez-Sanchez et al., 2000). Cysteine proteinases (CPs) have been implicated with lytic and adherence factors, thus aiding the hemolysis of erythrocytes as well as the adherence of *T. vaginalis* to the epithelial cells (Petrin et

al., 1998). CPs possesses the ability to degrade the host immunoglobulin and is pathogenic (Petrin et al., 1998). Study by Mendoza-Lòpez et al. (2000) explained the involvement of CP30, a cysteine proteinase in attachment to host epithelial cells. The attachment subsequently caused the degradation of proteins found on the female urogenital tract. Similarly, CPs was known to induce cell death in human vaginal epithelial cells (HVEC) as demonstrated through Cell Death ELISA assay and flow cytometry analyses (Sommer et al., 2005).

2.9.4 Contact-independent mechanisms and cell detaching factor

Contact-independent cytolytic mechanisms have been identified and major cytolytic effects have been related to substances released by the parasite in the culture medium (Ryan et al., 2011; Sommer et al., 2005). Therefore, contact-independent factors of *T. vaginalis* are also important in triggering pathogenesis (Bhatt et al., 1996). Contrary to contact-dependent mechanisms of *T. vaginalis* such as cytocidal and haemolytic properties, Pindak et al. (1993) proved that cell death of the host cells still occurs without the addition of erythrocytes through the production of acidic metabolites and other factors by the living parasite alone which causes apoptosis of the host. One of these factors is known as cell detaching factor (CDF), a cell-free product of *T. vaginalis* with significant cytopathic effect (Petrin et al., 1998). CDF is 200 kDa glycoprotein, heat and acid labile and exert a pH dependent activity (Sood & Kapil, 2008). This is clinically important since the normal pH of vagina is 3.9 to 4.2 but becomes more than pH 5 during trichomoniasis, suggesting that rise of vaginal pH during infection contributes to pathogenesis (Sood & Kapil, 2008; Adegbaju & Morenikeji, 2008). One earlier study has successfully isolated CDF from *T. vaginalis* and explained a trypsin-like activity in the parasite that can disrupt the monolayer of cells (Garber et al., 1989). Levels of CDF have also been shown to exert different severity of clinical symptoms of vaginitis with high concentration of CDF having chronic infection (Petrin et al., 1998).

2.9.5 Phagocytosis

Extensive studies using light and electron microscopy have demonstrated the phagocytic activity of *T. vaginalis* as being able to efficiently ingest and degrade lactobacilli, vaginal and cervical epithelial cells, leukocytes, erythrocytes (Rendón-Maldonado et al., 1998), yeast cells (Pereira-Neves & Benchimol, 2007), prostatic cell (Vazquez-Carrillo et al., 2011) and spermatozoids (Benchimol et al., 2008; Midlej & Benchimol, 2010). Although the exact mechanism has not been fully elucidated, however two types of phagocytic pathways have been described by Pereira-Neves and Benchimol (2007) based on their study on phagocytosis of *T. vaginalis* on yeast cells. These pathways include extension of pseudopodia towards target cells and sinking process without the extension of membrane cell of *T. vaginalis* (Figueroa-Angulo et al., 2012; Pereira-Neves & Benchimol, 2007). Amoeboid morphology is a significant structure observed during phagocytosis process which can be highly virulent as observed by few investigators (Pereira-Neves & Benchimol, 2007; Rendón-Maldonado et al., 1998). Therefore, it can be concluded that phagocytosis contributes to the virulence mechanism of *T. vaginalis* apart from other mechanisms used to acquire iron, lipids, nucleotides and relevant nutrients (Figueroa-Angulo et al., 2012).

2.9.6 Immune evasion mechanisms

Chronic vaginal infections and constant changing microenvironment encountered during the menstrual cycle enabled *T. vaginalis* to evolve defensive system against host immune responses using a variety of immune evasion mechanism (Figueroa-Angulo et al., 2012). *T. vaginalis* was able to overcome the host immune system using complement avoidance strategy (Adegbaju & Morenikeji, 2008; Petrin et al., 1998). This means that *T. vaginalis* take advantage on minimal complement

situation present in infected areas such as cervical mucus surrounded with deficiency in complement substances (Petrin et al., 1998).

To date, iron was found as a major contributing attribute in complement resistance of *T. vaginalis* (Petrin et al., 1998). The resistance to complement iron-rich conditions considerably induces microbial pathogenesis (Petrin et al., 1998). In a study conducted by Ryu and Min (2006), *T. vaginalis* was noted to exhibit cell-capping mechanisms in which antibody-induced membrane antigen modulated by the parasite can resist host immune responses.

Host humoral immune responses to *T. vaginalis* are also known to play a significant role as determinant of virulence (Malla, 2012). IgG, IgM and IgA are human immunoglobulins which can be easily degraded by the cysteine proteinases secreted by the parasite thus allowing the parasite to survive antibody response (Min et al., 1998). *T. vaginalis* also can coat itself with host plasma proteins, disallowing host immune system from recognizing the parasite as foreigners (Malla, 2012). The continuous release of antigens also may neutralize antibody or cytotoxic T lymphocytes, thus inhibiting specific anti-*T. vaginalis* defense mechanisms by host immune system (Alderete & Garza, 1985). Identification of antigenic proteins on the surface of *T. vaginalis* has also been suggested to stimulate immune responses by human cells (Lee et al., 2012).

Other invasion mechanisms applied by *T. vaginalis* includes molecular mimicry mechanism by decorating the parasite membrane with molecules homologous to host proteins (Alderete et al., 2001), induction of neutrophil recruitment via interleukin pathways in response to activation by live *T. vaginalis* (Cudmore et al., 2011*)* and surface immunogens activity (Petrin et al., 1998).

2.10 Predisposition of *T. vaginalis* infection to other complications

Growing incidence of infections with *T. vaginalis* reversed the initial acknowledgement as minor STD into major STD based on the increasing number of associated complications (Schwebke & Burgess, 2004; McClelland, 2008) with trichomoniasis. Examples of these complications include preterm delivery, low birth weight, premature rupture of membranes, acquisition of HIV, tendency to cervical cancer, pelvic inflammatory disease and infertility (Schwebke & Burgess, 2004; Petrin et al., 1998). Among these, predisposition to HIV and cervical cancer became the highly concerned associated diseases due to the significant health drawbacks it sets to *T. vaginalis* infected patient.

2.10.1 *T. vaginalis* and cervical cancer

Cervical cancer is classified as one of the most common malignant disease worldwide (Yap et al., 1995). Many of the earlier studies correlated human papillomavirus (HPV) as the major risk factor for cervical neoplasia (Viikki et al., 2000). However, it has been clearly proved by recent studies that *T. vaginalis* can also be major contributor for this disease. One of them is the cohort study conducted to determine other predictors beside HPV in causing cervical cancer (Viikki et al., 2000). The study proposed two possible risk determinants of subsequent cervical neoplasia with *T. vaginalis* as the major contributor to cervical cancer. It has also been reported that live *T. vaginalis* has been found to induce higher degree of immunosuppression by the more virulent strains suggesting the possible activation of carcinogenic viruses (Yap et al., 1995).

One possible mechanism identified in *T. vaginalis* infections progress into cervical cancer is that the parasite adheres to the wall of cervicovaginal cells and

through an effective enzyme systems, it interferes with host metabolism and generates toxins which finally leads to cell necrosis (Pustan et al., 2010).

A prospective study conducted by Yap et al. (1995) revealed high titre of antibodies in serum of patients with invasive cervical carcinoma marking a significant association of *T. vaginalis* infection with cervical cancer. Similarly, Zhang et al. (1995) was able to prove the association of *T. vaginalis* with cervical cancer among women subjects in China although the proportion rate was low. Afzan (2011b) were also able to screen for *T. vaginalis* from clinical patients in Malaysia with 0.86 % out of 1.58 % were diagnosed with cervical intraepithelial neoplasia.

2.10.2 *T. vaginalis* and acquisition of HIV

T. vaginalis has emerged as a co-factor for HIV transmission regardless of gender (Coleman et al., 2013). Almost all the studies conducted mentioned the close association of *T. vaginalis* with HIV infection. Four potential mechanisms have been identified for increased susceptibility for predisposition to HIV which includes (Coleman et al., 2013);

a. Recruitment of HIV target cells such as $CD4^+$ T cells to the genital tract as a result of the host immune response to *T. vaginalis.*
b. Degradation of HIV-protective factors such as secretory leukocyte protease inhibitor.
c. Direct and indirect cytotoxic effects of the parasite itself.
d. Disrupted or abnormal vaginal flora (Cudmore et al., 2011).

Cell-detaching factor is a contact-independent mechanism of *T. vaginalis* that aids transmission of HIV by releasing epithelial cells from tissues and weakening the defense barrier against HIV invasion (Coleman et al., 2013). Besides, the discharge of punctuate micro hemorrhages in infected mucosal genital tract tissue also known to aid the transmission by acting as portal for HIV entry (Coleman et al., 2013).

Studies by Van Der Pol et al. (2008) and Miller et al. (2008) highlighted that *T. vaginalis* infection may be epidemic in populations at risk for HIV-1 transmission in resource limited countries and in the developed world taking the complications to a general population cohort. Based on a review study conducted, it was evident that amplifying effect of *T. vaginalis* is fundamental in increasing the HIV infections among African-Americans especially those with low-income (Shafir et al., 2009).

Rates of trichomoniasis associated-HIV infections were prominently higher in women. A recent review study confirmed the interactions of trichomoniasis and HIV by providing strong evidence on the increased risk of transmission and acquisition of HIV among women (Kissinger & Adamski, 2013). The association of trichomoniasis with HIV transmission was also seen among men (Soper, 2004). Hobbs et al. (1999) performed a prevalence study among Malawian men and concluded that symptomatic inflammatory trichomoniasis may cause the increased incidence of HIV excretions in semen.

Co-infection is another phenomenon in which HIV-positive patients become prevalent to other infections such as trichomoniasis and bacterial vaginosis. Incidence of co-infection with HIV and *T. vaginalis* in women has been reported to vary between 16 % to 30 % (Allsworth et al., 2009; Gatski et al., 2011). However, it is difficult to estimate the rate of co-infection in men due to asymptomatic characteristics of *T. vaginalis* infection (Cudmore et al., 2011).

2.11 Growth requirements and survival of *T. vaginalis*

Studies on the nutritional requirements of *T. vaginalis* progressed with the advent of axenic culture by Trussell in 1940 (as cited in Clark & Diamond, 2002). Axenic culture of *T. vaginalis* incorporates antifungal and antibacterial antibiotics into medium to prevent growth of any other metabolizing cells (Clark & Diamond, 2002). It is an obligate parasite, lacking the ability to synthesize many macromolecules de novo

such as purines, pyrimidines and lipids (Petrin et al., 1998; Sood & Kapil, 2008). In vagina, these nutrients are acquired through secretions or via phagocytosis of host and bacterial cells (Petrin et al., 1998; Sood & Kapil, 2008). However, under *in vitro* growth conditions, these nutrients are supplemented in the culture media to ensure efficient growth of *T. vaginalis* (Kostara et al., 1998; Sood & Kapil, 2008).

Being a fastidious organism, *T. vaginalis* can only be cultured in medium containing sufficient growth requirements such as yeast extract, trypticase soy broth, maltose, minerals and vitamins (Wang, 2001). Serum is an important supplement in the culture media as it contains lipids, fatty acids, amino acids and trace metals (Sood & Kapil, 2008). Examples of axenic medium used for the successful growth of *T. vaginalis* includes Diamond medium (Schmid et al., 1989), Modified Hollander medium (Afzan, 2011b), Feinberg and Whittington medium (Kostara et al., 1998), TYI-33 and YI-S medium (Clark & Diamond, 2002).

T. vaginalis is a microaerotolerant flagellate (Sehgal et al., 2012) that tolerates low level of oxygen and are anaerobic (Guschina et al., 2009). An optimum growth temperature suggested by Trussell and Johnston (1944) which has been maintained to date is 35ºC to 37ºC. Outside the host, the parasite can survive up to six to 24 hours in swimming pool water, in urine and semen whereas lasts up to 30 minutes when exposed to air ("*Trichomonas vaginalis"*, 2010). *T. vaginalis* can grow over a wide range of pH (Wang, 2001) with optimum level between pH 6 to 6.3 (Sood & Kapil, 2008).

2.12 Diagnosis

Diagnosis for trichomoniasis cannot be readily made solely based on clinical signs and symptoms since *T. vaginalis* holds a broad spectrum of infection with several STDs possessing similar clinical features (Sood & Kapil, 2008). Hence, laboratory diagnosis methods are significant in detecting the infection accurately so that appropriate treatment and management of the disease can be applied (Petrin et al.,

1998). These diagnostic tests varies from some old techniques such as direct microscopic examination and Pap smear to newer techniques such as culturing method and RNA analysis up to recent methods such as rapid antigen and nucleic acid amplification (Harp & Chowdhury, 2011). Different investigators use various kinds of diagnostic tools depending on the resource and cost availability therefore presenting a range of sensitivity and specificity to diagnose trichomoniasis (Patel et al., 2000). Significance of rapid diagnosis of *T. vaginalis* infection has been reported by Mitchell and Hussey (2010) using a case of vulval ulceration in a 41-year old female that remained unnoticed for five years. In male, urine specimens were used as diagnostics samples whereas in females, endocervical and vaginal swab specimens were more common (Harp & Chowdhury, 2011).

2.12.1 Direct microscopy

Preparation of saline wet mount from the vaginal swab or urine specimen and screening them under light microscopy is one of the conventional methods used. It is one of the cost-effective and prompt diagnostic methods known. Although highly specific (Wiese et al., 2000), however, this techniques became less reliable due to the low sensitivity (30 % to 80 %) and requirement of trained microscopists reported (Adu-Sarkodie et al., 2004). Direct microscopy observation (Figure 2.7) requires visualization of viable, motile trophozoite indicating that the specimens must be examined immediately (Hobbs & Sena, 2007).

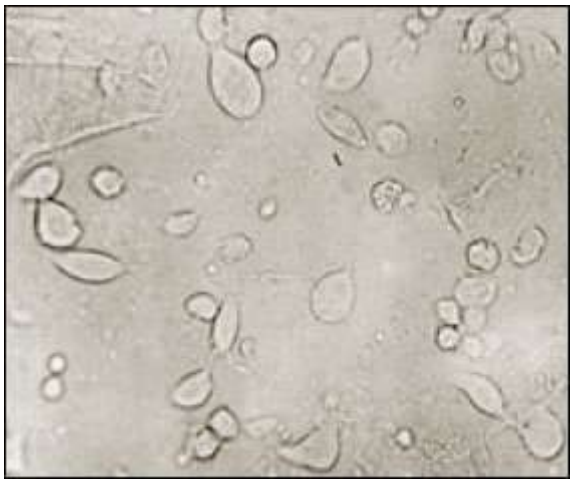


Figure 2.7: Example of wet mount preparation of *T. vaginalis*. Magnification 400X. (Ng, 2007).

Most of the comparative studies done to evaluate the efficiency of different diagnostic methods revealed direct microscopy as less preferred method mainly due to the low sensitivity in detecting *T. vaginalis* in large number of women populations (Jamali et al., 2006; Fernando et al., 2011; Sadek & Gammo, 2012). Most men are asymptomatic for trichomoniasis and direct microscopy diagnosis has been reported to produce a less productive results compared to other latest techniques used (Chapin, 2013). Nevertheless, sensitivity of wet mount examination has been reported to increase when coupled with other diagnostic techniques such culture method (Cohen et al., 2006; Patullo et al., 2009; Patil et al., 2012).

2.12.2 Staining method

Various kinds of staining methods have been implied to detect *T. vaginalis* such as using Gram stain (Stefanski et al., 2010) Giemsa stain (Akujobi & Ojukwu, 2006; Radonjic et al., 2006; Karaman et al., 2008), Papanicolaou (PAP) smear stain (Karaman et al., 2008), periodic acid-Schiff stain (Sood & Kapil, 2008), fluorescent staining (Van Der Schee et al., 1999; Radonjic et al., 2006)and Modified Field's stain (Afzan et al., 2010). Despite the relative easiness of performing staining diagnostic method, yet it

remained as the least preferred due to the time-consuming and tediousness of preparing the specimens. Figure 2.8 illustrates examples of Gram stain, Modified Field Stain and Giemsa stain performed by Afzan et al. (2010) for diagnosis of *T. vaginalis* in infected women.

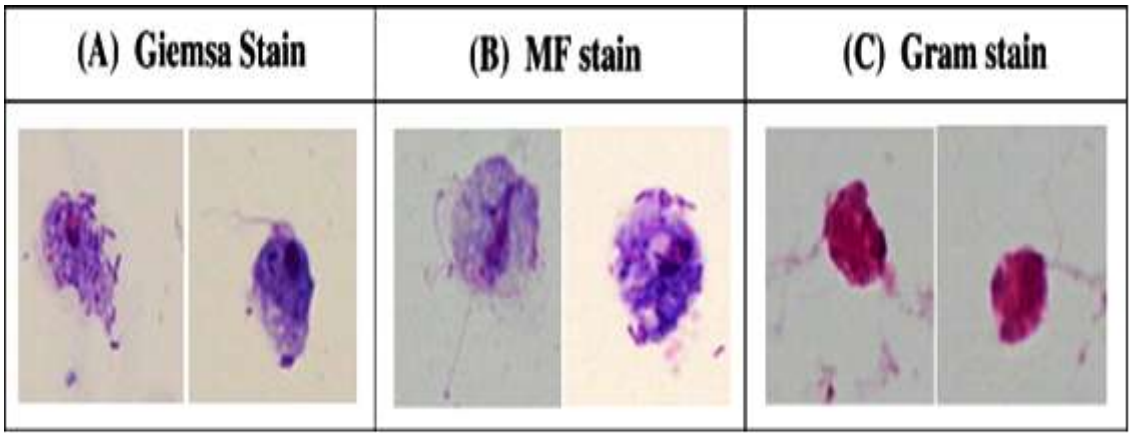


Figure 2.8: Three different types of staining method; A) Giemsa stain B) Modified Field's stain C) Gram stain performed for the diagnosis of *T. vaginalis*. Magnification x400 (Afzan et al., 2010).

A comparative study between wet mount, Giemsa stain and culture method of vaginal swabs found that the culture method provides more positive results of 9.04 % with Giemsa staining giving only 6.7 % of positive results (Akujobi & Ojukwu, 2006). However, in resource limited countries such as Sri Lanka, Giemsa stain was known to give 100 % sensitive and 99 % specific results compared to culture and wet smear examination (Fernando et al., 2011). Radonjic et al. (2006) reported that the sensitivity of Giemsa and acridine orange staining was 52 % and 71 % respectively compared to wet mount examination. This study concluded that although staining method is easier to perform, however it requires fluorescence microscopy and longer preparation time.

2.12.3 Culture method

The broth culture method is known as the gold standard and has been recognized as the most sensitive for routine diagnosis of *T. vaginalis* (Malla, 2012; Petrin et al.,

1998). InPouch (Hobbs et al., 2006; Sadek & Gammo, 2012) and cell culture technique (Bhatt et al., 1996) has also been implemented with added advantages such as being portable and requires small volume of inoculums respectively (Malla, 2012; Sood & Kapil, 2008). However, the high cost involved, greater risk of contamination and inconvenience for rapid diagnosis in using these two techniques made broth culture method as the most reliable method (Malla, 2012; Garber, 2005). In men, optimal sensitivity is said to be obtained using culture technique using urethral swab and urine specimen (Sood & Kapil, 2008).

Examples of broth cultures used includes Diamond's Trypticase-yeast medium (Radonjic et al., 2006), Kupferg medium (Jamali et al., 2006; Van Der Schee et al., 1999) and Hollander medium (Afzan, 2011). In a study to detect *T. vaginalis* by different methods in women from Iraq found that diagnosis with Diamond modified culture medium yielded higher rate of infection (Al-Saeed, 2011). Culture method is known to detect as few as 1-10 organisms in a specimen and sensitivity range is between 92 to 95 % (Wang, 2001). One limitation of culturing technique is the need to incubate samples for several days prior to detection which is time-consuming (Lewis, 2010). In a study by Akujobi and Ojukwu (2006), cultivation technique was reported as most sensitive though costly and slow. The study also suggested that the culture method can be used to complement wet mount method in order to achieve higher rate of *T. vaginalis* isolation.

Another type of culture method used was solid medium which has also been proved to be effective. Example is the use of Modified Columbia Agar which was able to identify 98 % of positive samples and is categorized as reliable, sensitive, time-saving and suitable medium to detect infection in asymptomatic and symptomatic individuals (Stary et al., 2002).

2.12.4 Molecular method

In conjunction to the limitations possessed by wet mount, staining and culture methods, a more specific and improved sensitivity molecular assays for the detection of *T. vaginalis* DNA known as Polymerase Chain Reaction (PCR) was developed (Caliendo et al., 2005). Review of reports indicated that DNA detection in clinical samples by PCR yielded a fast and higher sensitivity results compared to wet mount and culture (Schwebke & Burgess, 2004). Diagnosis of trichomoniasis by PCR has been a widely accepted method based on the many clinical studies performed (Madico et al., 1998; Van Der Schee et al., 1999; Lawing et al., 2000; Jamali et al., 2006; Valadkhani et al., 2010; Lee et al., 2012). Trichomoniasis detection in males has also been improved with the use of PCR methods (Schwebke & Lawing, 2002; Seña et al., 2007; Lee et al., 2012).

In a study, 100 % sensitivity and 99 % specificity in detecting *T. vaginalis* from vaginal swabs was achieved using Real-time PCR compared to culture method, suggesting the reliability of molecular method compared to conventional diagnosis (Caliendo et al., 2005). In a case study performed by Bellanger et al. (2008) for a trichomonal empyema adult woman and *T. vaginalis* infected infant, the PCR method was known to complement the weak sensitivity of wet mount and low viability of *T. vaginalis*by providing a rapid and consistent identification of the trichomonad species. Wendel et al. (2002) proposed PCR as an ultimate solution to diagnose *T. vaginalis* infection in high-risk group women especially those with perinatal morbidity and HIV-positive. One potential strength of PCR is its usefulness in mass screening of trichomoniasis (Patel et al., 2000).

Diverse PCR types have been implemented for a better and improved diagnosis of *T. vaginalis* infections. This includes PCR–enzyme-linked immunosorbent assay (PCR-ELISA) used for detection of *T. vaginalis* in urethral swabs, urine, and semen of

male sexual partners of women (Hobbs et al., 2006). Others include random amplified polymorphic DNA analysis (RAPD) (Jamali et al., 2005) to detect *T. vaginalis* DNA polymorphism, multiplex PCR (Diaz et al., 2010) and restriction fragment length polymorphism (RFLP) (Crucitti et al., 2008).

Many different primers have been developed with regard to refining and improving the sensitivity of PCR method (Schwebke & Burgess, 2004; Swygard et al., 2003). Although obtaining significant results were assertive with PCR methods, however high costs and technical complexity have limited the method to research settings (Soper, 2004).

2.12.5 Other diagnostic methods

Additional diagnostic methods which have been invented and evaluated to enhance diagnosis of *T. vaginalis* includes fluroscent taxoid FLUTAX (Lecke et al., 2003), latex agglutination (Adu-Sarkodie et al., 2004), antigen-based diagnostic tests (Soper, 2004), Food and Drug Adminisration (FDA)-approved point-of-care tests (i.e. OSOM, rapid antigen test and Affirm VP III, nucleic acid probe-hybridization) (Bachmann et al., 2011; Hobbs & Sena, 2007), XenoStrip-Tv rapid assay (Kurth et al., 2004), antibody based technique (i.e. complement fixation, hemagglutination, gel diffusion, fluorescent antibody and ELISA) (Garber, 2005), and Transcription-Mediated Amplification-Based Analyte-Specific-Reagent Testing (Munson et al., 2008). Huppert et al. (2007) reported that the sensitivities of rapid antigen tests and transcription-mediated assay were comparable with 92 % and 97 % respectively making these tests superior to wet mount examination. Although rapid tests can overcome the weakness of *in vitro* culture methods, however, these tests are seldom used in clinical settings due to the limited availability of sensitivity and specificity data (Wang, 2001).

2.13 Treatment and Control

Knowledge of clinical features, the virulence mechanisms, diagnosis methods and the epidemiological distributions of *T. vaginalis* enhance the effective treatment and management of this infection. Introduction of nitroimidazoles in 1960 for the systemic treatment of trichomoniasis became the turning point to the therapeutic approach of this infection (Wang, 2001). Derivative of nitroimidazoles includes metronidazole, tinidazole, ornidazole and secnidazole have been assessed for therapeutic effects in *T. vaginalis* infections (Ryu & Min, 2006). Among these, metronidazole has been widely used in the treatment (Afzan, 2011a) especially in the United States (Soper, 2004) since it is highly effective and approved by WHO (1995) (Ryu & Min, 2006; Workowski & Berman, 2010) Metronidazole is a 5-nitroimidazole, a heterocyclic compound derived from *Streptomyces* antibiotic azomycin (Sood & Kapil, 2008). Metronidazole enters trichomonads by passive diffusion (Sood & Kapil, 2008; Ryu & Min, 2006).

The recommended regimen of metronidazole consumption by CDC is 2 g orally in a single dose or alternatively 500 mg orally twice a day for seven days (Workowski & Berman, 2010). If an infected woman's male partner is also treated, then the cure rates in women were reported similar, approximately 95 % with both regimens (Ackers, 2001). However, if the male partner is not treated, the cure rates were lower with single-dose regimen (Ackers, 2001). Despite the excellent cure rate, treatment failure is often encountered probably due to non-compliance or reinfection with *T. vaginalis* (Petrin et al., 1998). However, extensive studies proposed that the main cause of the treatment failure is due to resistance mechanisms of *T. vaginalis* strains to metronidazole (Petrin et al., 1998).

Resistance towards metronidazole by *T. vaginalis* strains is an emerging problem with this drug (Wendel & Workowski, 2004; Harp & Chowdhury, 2011). Failure of drug resistance by *T. vaginalis* have been clinically studied by Dunne and

team (2003) giving other prospective therapeutic agents as an alternative treatment for the infection. Likewise, Meri et al. (2000) reported three cases of metronidazole-resistance of *T. vaginalis* in Finland and suggested a relatively simple method of identifying resistant strains through *in vitro* susceptibility testing. According to a source, CDC estimated that 5 % of clinical isolates of *T. vaginalis* demonstrated certain degree of resistance towards metronidazole (Swygard et al., 2003). In a study, metronidazole treatment of asymptomatic pregnant women infected with *T. vaginalis* was found to successfully eliminate the parasite but could not prevent preterm delivery suggesting that this drug may not be appropriate for asymptomatic women (Klebanoff et al., 2001).

Similar to metronidazole, another derivative of 5-nitroimidazole known as tinidazole has also been widely accepted for treatment of trichomoniasis (Schwebke & Burgess, 2004). *T. vaginalis* strains that are resistant to metronidazole have been said to react effectively with tinidazole (Cudmore et al., 2004) with a plasma elimination half-life of metronidazole (Harp & Chowdhury, 2011). Moreover, tinidazole penetrates better into male reproductive tissues compared to metronidazole (Harp & Chowdhury, 2011) making it a better choice of treatment. Nonetheless, *T. vaginalis* is also known to exhibit resistance towards tinidazale but relatively in smaller number, 0.56 % compared to 9.6 % by metronidazole (Schwebke & Barrientes, 2006).

Potential side effects such as metallic taste, headache, insomnia, drowsiness, dry mouth and nausea have been reported with the use of metronidazole or tinidazole (Moore, 2007; Cudmore et al., 2004). Alternative therapeutic approaches in treating *T. vaginalis* infections include nonoxynol-9 vaginal derivatives, clotrimazole derivatives, acetarsol derivatives, sulfimidazole, vaginal paromomycin preparations, combination of borad-spectrum antibitiocs and metronidazole (Cudmore et al., 2004; Lewis, 2010) as well as natural products (Gehrig and Efferth, 2009).

Treatment and control is an important aspect in the study *T. vaginalis* because untreated infections may remain "silent" leaving millions of men and women at increased risk of reproductive health complications and HIV transmission (Van Der Pol, 2007). Control of risk associated factors such as smoking, frequent change of sexual partner, sharing of needles and other contagious materials must be taken care to prevent severity of the infections.

CHAPTER 3

METHODOLOGY

3.1 Source of *T. vaginalis* isolates

This study was conducted using the samples collected by Afzan (2011b). The samples had been screened and identified for positive isolates. The screening was based on routine PAP smears and vaginal swabs of females seen at the Obstetric and Gynecology clinic. It was collected from patients from a local hospital complaining of vaginal discharges and itching. Actively growing *T. vaginalis* from CN isolates and NCN isolates were obtained from the culture maintained in Department of Parasitology, University Malaya. A total of three NCN isolates (NCN2 –NCN4) and six CN isolates (CN1 – CN6) were used in this study. NCN1 isolate died while maintaining in the *in vitro* culture. The identity of each isolates and their respective symptoms as obtained by Afzan (2011b) is shown in Appendix A.

3.2 *In vitro* cultivation for maintenance of isolates

The positive isolates were maintained axenically in Hollander medium (Appendix B) supplemented with 10% heat inactivated Horse serum (Gibco, Life Technologies) and 2 % Penicillin-Streptomycin-Neomycin (Gibco, Life Technologies) antibiotic mixture. All the isolates were grown in 15 ml screw-capped falcon tubes and incubated at 37ºC. Sub-culturing were performed every three days once by adding the Hollander medium up to 10 ml per isolate and screened once a week using light microscope to ensure the viability of the parasite. Isolates with active motile trophozoites were used for the phenotypic analysis of amoeboid forms in this study.

3.3 Trophozoites counts of *T. vaginalis*

The parasites of each isolate from day three culture were centrifuged at 2000 rpm for 10 minutes and the supernatant was discarded up to 1 ml. The pellets were then suspended well before the count was taken. 10 µl of the suspension was mixed with 10 µl of 0.4 % Trypan blue dye exclusion (Sigma-Aldrich Corp. USA). The trophozoite count was done using haemacytometer chamber (Improved Neubauer, Hausser Scientific). The chamber was then observed under light microscope at 400X magnification prior to counting. The blue dye acted as viability indicator and only viable cells that did not take up trypan blue stain was counted. Trophozoites were identified by observing their motile movements and twitching flagella.

3.4 Growth profiling of amoeboid forms of *T. vaginalis*

Prior initiating the growth profiles of amoeboid forms of *T. vaginalis*, initial counts of trophozoites were performed as mentioned in **Section 3.3**. Based on the numbers of trophozoite counted, a final inoculum size of 1 x 10^4 *T. vaginalis* per ml was made from the day three culture and inoculated into 3 ml culture tubes containing Hollander medium supplemented with 10 % heat inactivated horse serum and 2 % Penicillin-Streptomycin Neomycin antibiotic mixture. All cultures were kept in airtight screw-capped tubes and incubated at 37ºC until all the cells became non-viable. All experiments were done in triplicates. Amoeboid forms were identified by observing their non-motile movements with none or inactive flagella and delicate outer layer showing pseudopodia-like cytoplasmic extensions. Trophozoites and pseudocysts forms were excluded in this particular growth profile study. Growth profile and the percentage of amoeboid forms in the isolates were generated. Data significance were analysed using SPSS. The parasite concentration per ml (a) and initial inoculum concentration per ml (b) was calculated according to the given formulae;

(a) ***T. vaginalis*** **concentration (*T. vaginalis*/ml):**

Average number of trophozoites in 5 squares x dilution factor x 10^4

Where;

Dilution factor = 2 (1:1 dilution with trypan blue)

10^4 = count in 10 squares from large four squares

(b) Initial inoculum concentration (µl/ ml):

$$\frac{x}{1000} = \frac{1 \times 10^4}{y}$$

Where;

x = Initial inoculum concentration (µl/ ml)

y = *T. vaginalis* concentration (*T. vaginalis*/ml)

3.5 Growth profiling of trophozoite forms of *T. vaginalis*

Based on the number of trophozoites counted (**Section 3.3**), a final concentration of 1 x 10^4 *T. vaginalis/*ml was made from the day three culture and inoculated in 3 ml culture tubes containing Hollander medium supplemented with 10 % heat inactivated horse serum and 2 % Penicillin-Streptomycin Neomycin antibiotic mixture. All cultures were kept in airtight screw-capped tubes and incubated at 37ºC. Counts of viable trophozoites detected using trypan blue were recorded up to 11 days until all the parasites became non-viable. Any other morphology besides trophozoites was excluded in this specific growth profile study. All experiments were done in triplicates. Growth profiles of trophozoites in CN isolates and NCN isolates were generated. Data significance were analysed using SPSS. The parasite concentration per ml and initial inoculum concentrations per ml was calculated using the formula shown in **Section 3.4(a)** and **(b)**.

The parasite concentration was increased to 10^5 *T. vaginalis*/ml and the methods mentioned in **Section 3.5** were repeated for CN isolates to generate the growth profile of trophozoites.

3.6 Giemsa Stain method for detection of amoeboid forms

Clean glass slides were smeared with drop of the *T. vaginalis* culture sediment of all the isolates showing amoeboid forms as detected from the growth profile study in **Section 3.5**. The smears were air dried and fixed with methanol for 30 seconds and stained with Giemsa stain at (Merck) dilution rate of 1:1 following the protocol of Sivanandam and Joon-Wah (as cited in Afzan et al., 2010) and allowed to air dry at room temperature for 10 – 30 minutes. The completely dried slides were then rinsed with distilled water and allowed to dry to room temperature prior to mounting with DPX. The slides were observed under 400X magnification using a light microscope. Similarly, trophozoites detected from growth profile study in **Section 3.5** were stained with Giemsa for comparison with the amoeboid forms.

3.7 Modified Fields' Stain method for detection of amoeboid forms

A small drop of the culture sediment from all the isolates showing amoeboid forms as detected from the profile study in **Section 3.4** were smeared on clean glass slides. The smear was allowed to dry at room temperature for approximately 15-20 seconds. Subsequently, six drops of Fields' stain B (0.2 % solution of eosin Y (Sigma Aldrich) in methanol) and 10 drops of Fields' stain A (LabChem Sdn Bhd) were immediately added. The slides were tilted to right and left for 10 – 20 seconds until a golden scum appeared on the surface of the smear. The slides were then rinsed for two seconds under brisk stream of tap water and allowed to dry to room temperature and mounted using DPX. The slides were observed under light microscope at 400X

magnification. Similarly, trophozoites detected from growth profile study in **Section 3.5** were stained with Modified Fields' stain for comparison with amoeboid forms.

3.8 Assessing the effect of different stress conditions in triggering amoeboid forms

Three types of stress conditions were applied to the suspension culture as mentioned in the subsequent sub-methods to observe the formation of amoeboid forms in the positively detected isolates. The protocol to study the effect of these stress conditions were performed similarly as the growth profiling methods mentioned in **Section 3.3** and **Section 3.4** except for the parameters studied as described in the following sub-methods. The cultures were observed for amoeboid formation and numbers of viable amoeboid *T. vaginalis* were counted using the haemacytometer chamber until all the parasites became non-viable. All the following experiments were done in triplicates. The growth profiles were generated and analyzed.

3.8.1 Effect of different parasite concentrations on amoeboid formation

The protocol to study the effect of varying concentrations of *T. vaginalis* per ml on the development of amoeboid forms was similar as mentioned in **Section 3.4** except that the final inoculum size was made to 1 x 10^3 *T. vaginalis*/ml, 1 x 10^5 *T. vaginalis*/ml and 1 x 10^6 *T. vaginalis*/ml. Control in this specific growth profile study was the parasites concentration of 1 x 10^4 *T. vaginalis*/ml. All experiments were conducted in triplicates. The cultures were observed for amoeboid formation and numbers of viable amoeboid *T. vaginalis* were counted until all the parasites became non-viable using the haemacytometer chamber. The results obtained were recorded and data significance were analysed using SPSS.

3.8.2 Effect of different horse serum concentrations on amoeboid formation

The protocol to study the effect of varying the concentrations of horse serum on the development of amoeboid forms was performed as mentioned in **Section 3.4**. However, the concentration of horse serum added to the Hollander medium prior to culturing is varied from 1 %, 5 %, 10 %, 15 % and 20 %. The final inoculum size of *T. vaginalis* were maintained at 1 x 10^4 *T. vaginalis*/ml. Control in this specific growth profile study was the horse serum concentration of 10 %. All experiments were conducted in triplicates. The cultures were observed for amoeboid formation and numbers of viable amoeboid forms of *T. vaginalis* were counted until all the parasites became non-viable using the haemacytometer chamber. The results obtained were recorded and data significance were analysed using SPSS.

3.8.3 Effect of different metronidazole drug concentrations on amoeboid formation

The protocol to study the effect of metronidazole drug (Sigma Aldrich) on the development of amoeboid forms was performed as mentioned in **Section 3.4**. Different concentrations of metronidazole drug ranging from 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml and 0.0001 mg/ml were introduced into the culture containing the isolates. The final inoculum size was maintained at 1 x 10^4 *T. vaginalis*/ml. Control in this specific growth profile study was without the addition of metronidazole drug for each isolate. All experiments were conducted in triplicates. The cultures were observed for amoeboid formation and numbers of viable amoeboid *T. vaginalis* were counted until all the parasites became non-viable using the haemacytometer chamber. The results obtained were recorded and data significance were analysed using SPSS.

3.9 Transformational changes of *T. vaginalis* from trophozoites to amoeboid forms

The parasites of each isolate from day three culture were centrifuged at 2000 rpm for 10 minutes and the supernatants discarded up to 1 ml. The pellets were then suspended well before the count was taken. 10 µl of the suspension was mixed with 10 µl of 0.4 % Trypan blue dye exclusion and amoeboid count was done using haemacytometer chamber. The chamber is then observed under a light microscope at 400X magnification prior to counting. The blue dye acts as a viability indicator and only viable trophozoites that did not take up trypan blue stain were counted. Based on the number of parasites counted, a final inoculum size of 1 x 10^5 *T. vaginalis* per ml was made from the day three culture and inoculated in 3 ml culture tubes containing Hollander medium supplemented with 10 % heat inactivated horse serum, 2 % Penicillin-Streptomycin Neomycin antibiotic mixture. All cultures were kept in airtight screw-capped tubes and incubated at 37ºC. The transformational change that took place in terms of size and shape from trophozoites to amoeboid forms was measured and recorded every day until no amoeboid forms were observed using a light microscope.

3.10 Statistical analysis

Statistical analysis was performed using SPPS version 20 (IBM SPSS Statistics). Significance of trophozoites count between days and between CN isolates in 10^4 *T. vaginalis/*ml and 10^5 *T. vaginalis/*ml respectively were tested using multiple comparison method meanwhile Mann-Whitney Test was used to compare the average trophozoites count between 10^4 *T. vaginalis/*ml and 10^5 *T. vaginalis/*ml. The significance between days of average amoeboid forms in CN isolates (CN1 – CN6) was calculated using Dunnett's T3. Positive mean differences and the significance value were used to determine the days with higher counts. Mann-Whitney Test was used to determine the

significant difference in the number of amoeboid forms observed between 10^4 *T. vaginalis*/ ml and 10^5 *T. vaginalis*/ml. The effect of different concentrations of horse serum and metronidazole drug on amoeboid formation in suspension culture were analysed using Dunnett's T3 and Tukey HSD respectively. In all cases, results with $p < 0.05$ were considered as statistically significant.

CHAPTER 4

RESULTS

4.1 Light micrograph of amoeboid forms

Amoeboid forms of *T. vaginalis* are a unique and distinguishable morphological form of the parasite. Unlike the trophozoites and pseudocysts form of *T. vaginalis*, the amoeboid forms were observed with pseudopodia-like cytoplasmic extension surrounding the parasites, non-motile, none or inactive flagella, more inclusion–like bodies, flattened, bigger and irregular in shape under a light microscope (Fig. 4.1). The amoeboid forms were easily detected with their larger and non-motile morphology compared to actively swimming trophozoite forms. This shape was significant for the present study in order to obtain the targeted results.

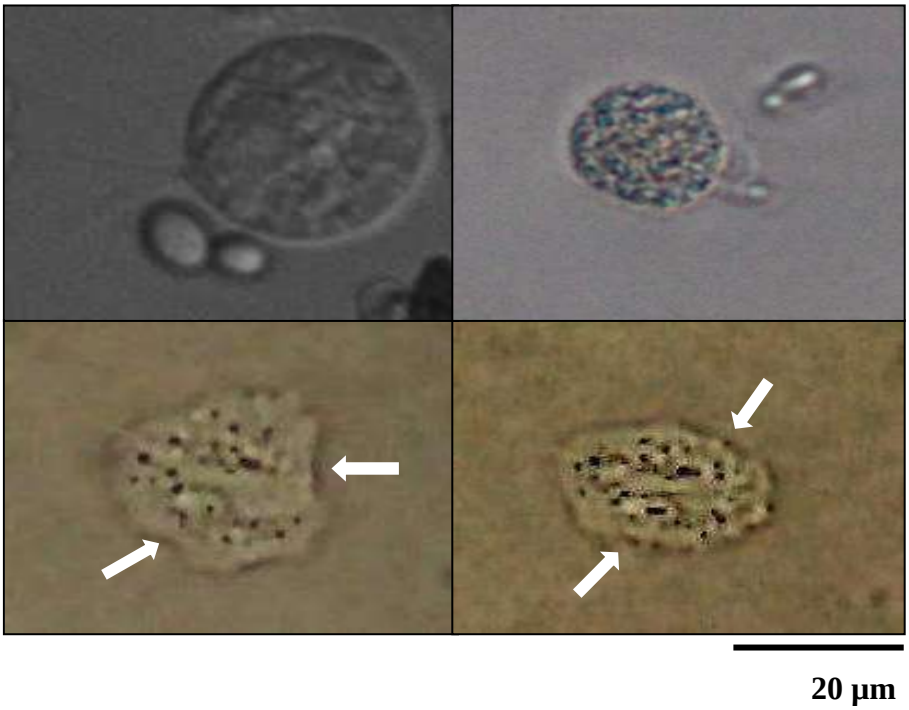


Figure 4.1: Light micrograph showing comparison of (a) round trophozoite form and (b) pseudocyst form with (c - d) amoeboid forms of *T. vaginalis* in suspension culture of CN isolates. Magnifications at 400X.
Note: Arrows indicate the pseudopodia-like cytoplasmic extensions in the amoeboid forms.

4.2 Growth profiles of amoeboid forms of *T. vaginalis*

The growth profile of amoeboid forms in CN and NCN isolates was performed for five consecutive days. Transformation of the motile trophozoites to non-motile amoeboid forms was only observed in CN isolates (Table 4.1) and none in NCN isolates. The growth pattern of amoeboid form of *T. vaginalis* followed a steady logarithmic growth phase from day 1 then peaked on day 3 and reached the death phase thereafter (Fig. 4.2a).

The average number of amoeboid forms was significantly different between the days ($p < 0.05$) with highest count on day three of the culture, $19.34 \pm 0.65 \times 10^4$ *T. vaginalis*/ml for all the CN isolates (CN1 – CN6). Data on the significant difference were presented in Table 4.2 meanwhile the raw data were shown in Appendix C. Amoeboid forms remained viable only for 96 hours in all the CN isolates. The total number of amoeboid forms observed in all the CN isolates from day 1 to day 5 was $28.7 \pm 7.93 \times 10^4$ *T. vaginalis*/ml with highest count of $19.34 \pm 0.65 \times 10^4$ *T. vaginalis*/ml on day 3. Lowest number of amoeboid forms were observed on day 1 with total count of $0.66 \pm 0.17 \times 10^4$ *T. vaginalis*/ml. Amoeboid forms were detected after 24 hours of incubation from isolate CN3 and isolate CN5 but in lower number of $0.33 \pm 0.58 \times 10^4$ *T. vaginalis*/ml respectively.

Based on percentages of amoeboid forms detected in each isolate (Fig. 4.2b), it can be concluded that all CN isolates had almost similar amount of these forms. The most number of amoeboid formations from day 1 to day 4 were observed in isolate CN6 with total average count of $6.00 \pm 1.69 \times 10^4$ *T. vaginalis*/ml (20.9 %), meanwhile the least was in isolate CN1 with an average of 13.9 %. Although every isolate reported different percentage of amoeboid counts, there was no significant preference among the CN isolates in triggering amoeboid forms ($p > 0.05$). Different types of the amoeboid forms of *T. vaginalis* were detected throughout the growth profile study in CN isolates

(Figure 4.3). Most of the amoeboid forms were observed with projections of cytoplasm, with none or inactive flagella and irregularly shaped.

Table 4.1: Average amoeboid count of *T. vaginalis* in CN isolates from day 1 to day 5

Isolates	**Average count (No of amoeboid x 10^4 *T. vaginalis* / ml)**					
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
CN 1	0.00	0.00	0.67 ± 0.58	**3.00** ± 1.00	0.33 ± 0.58	0.00
CN 2	0.00	0.00	1.00 ± 1.00	**3.67** ± 1.15	0.33 ± 0.58	0.00
CN 3	0.00	0.33 ± 0.58	1.33 ± 0.58	**2.67** ± 0.58	0.33 ± 0.58	0.00
CN 4	0.00	0.00	1.00 ± 1.00	**2.67** ± 2.08	0.67 ± 1.15	0.00
CN 5	0.00	0.33 ± 0.58	1.00 ± 1.00	**3.00** ± 0.00	0.33 ± 0.58	0.00
CN 6	0.00	0.00	0.67 ± 1.15	**4.33** ± 1.53	1.00 ± 1.00	0.00

Note: Values in bold and highlighted were the optimum amoeboid forms obtained on day three culture.

Table 4.2: Significance analysis between days using Dunnett T3 for average amoeboid forms of *T. vaginalis* in CN isolates at 10^4 *T. vaginalis*/ml.

Multiple comparisons

Dependent Variable: Average amoeboid count x 10^4 *T. vaginalis*/ ml

Dunnett T3

(I) Days of count	(J) Days of count	Mean difference (I – J)	Std. error	Sig.	95% confidence interval	
					Lower bound	Upper bound
Day 3	Day 1	3.11333*	.27577	**.000**	1.9849	4.2417
	Day 2	2.27833*	.28548	**.001**	1.1609	3.3958
	Day 4	2.66833*	.28941	**.000**	1.5522	3.7844
	Day 5	3.22333*	.26685	**.000**	2.0728	4.3739

*. The mean difference is significant at the 0.05 level

Note: Comparison of average amoeboid form on day 3 with the rest of the days yielded all positive values with p-value less than 0.05 (bold). The 95 % confidence interval does not contain the value 0. Thus, there is a significant difference of average amoeboid count between days with day 3 having significantly higher count.

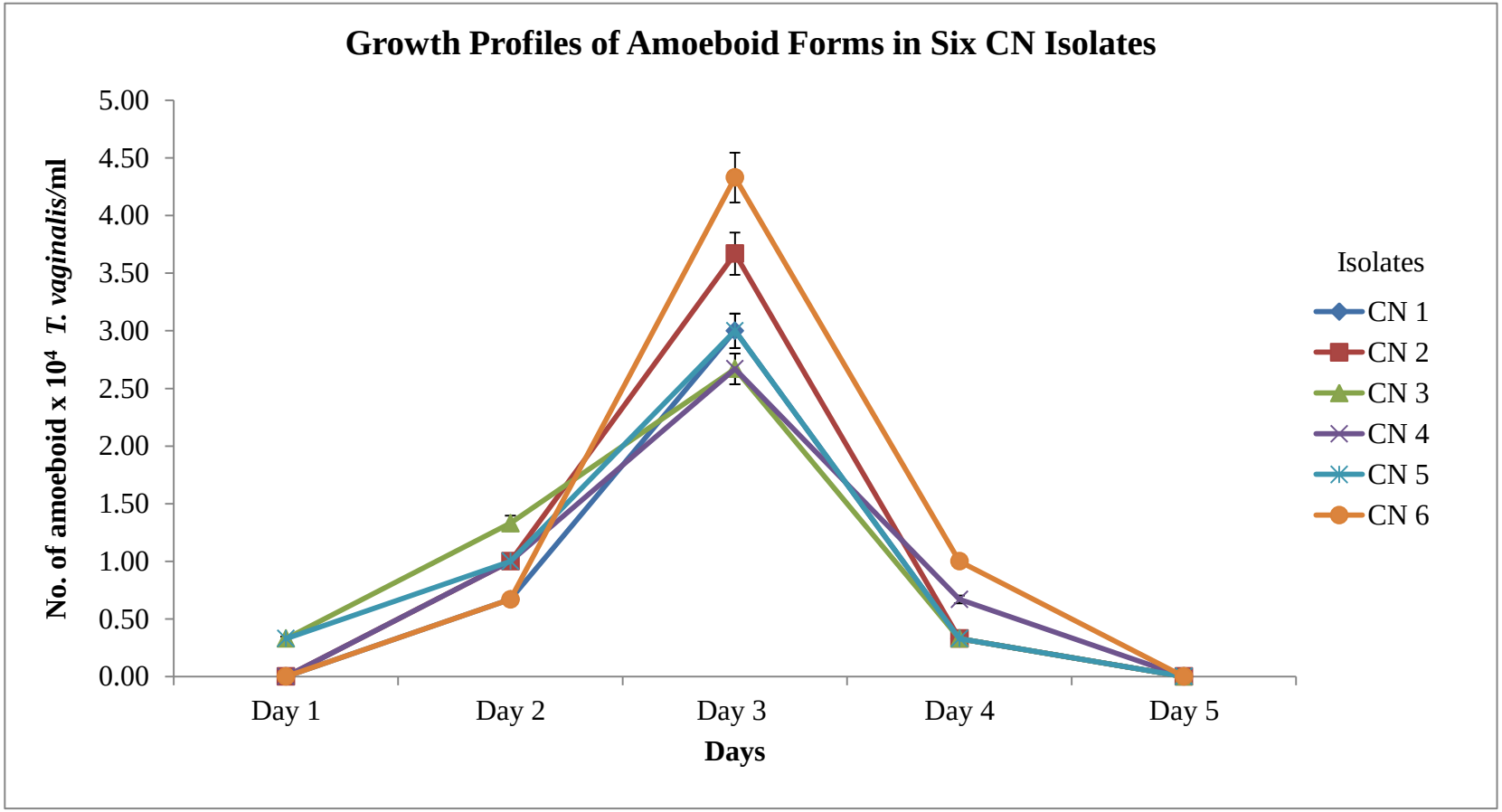


Figure 4.2a: Growth profiles of amoeboid forms of *T. vaginalis* in CN isolates (CN1 – CN6) at 10^4 *T. vaginalis*/ml inoculated in Hollander medium supplemented with 10 % Horse serum and 2 % Penicillin-Streptomycin Neomycin antibiotic mixture with optimum growth on day 3 of culture for all CN isolates.
Note: The values were expressed as average ± S.D.

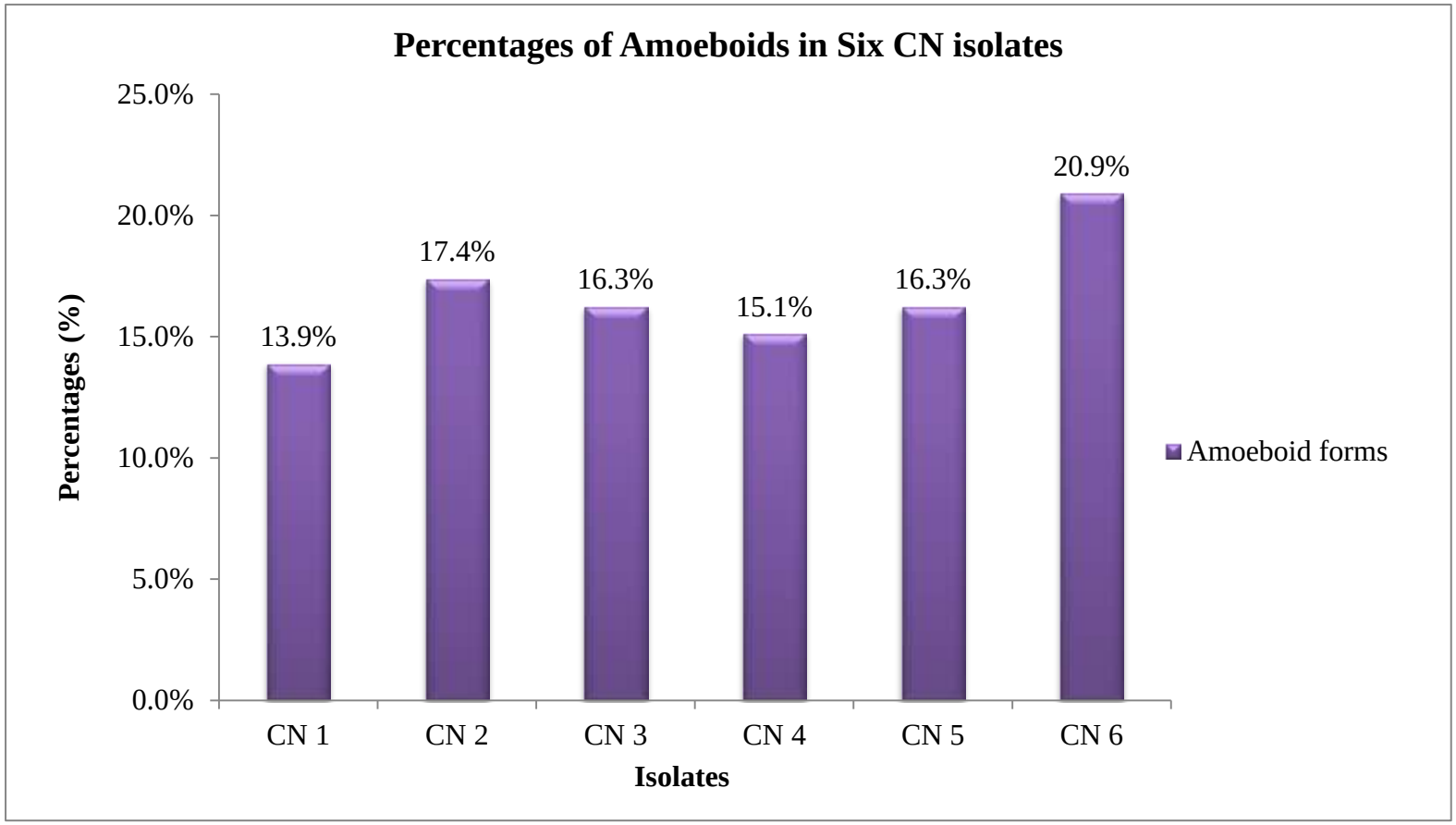


Figure 4.2b: Percentages of amoeboid forms of *T. vaginalis* in CN isolates (CN1 – CN6) grown in Hollander medium from day 1 to day 4 of culture. Highest percentage of amoeboid forms was observed in isolate CN6 which had a total of 20.9 %.

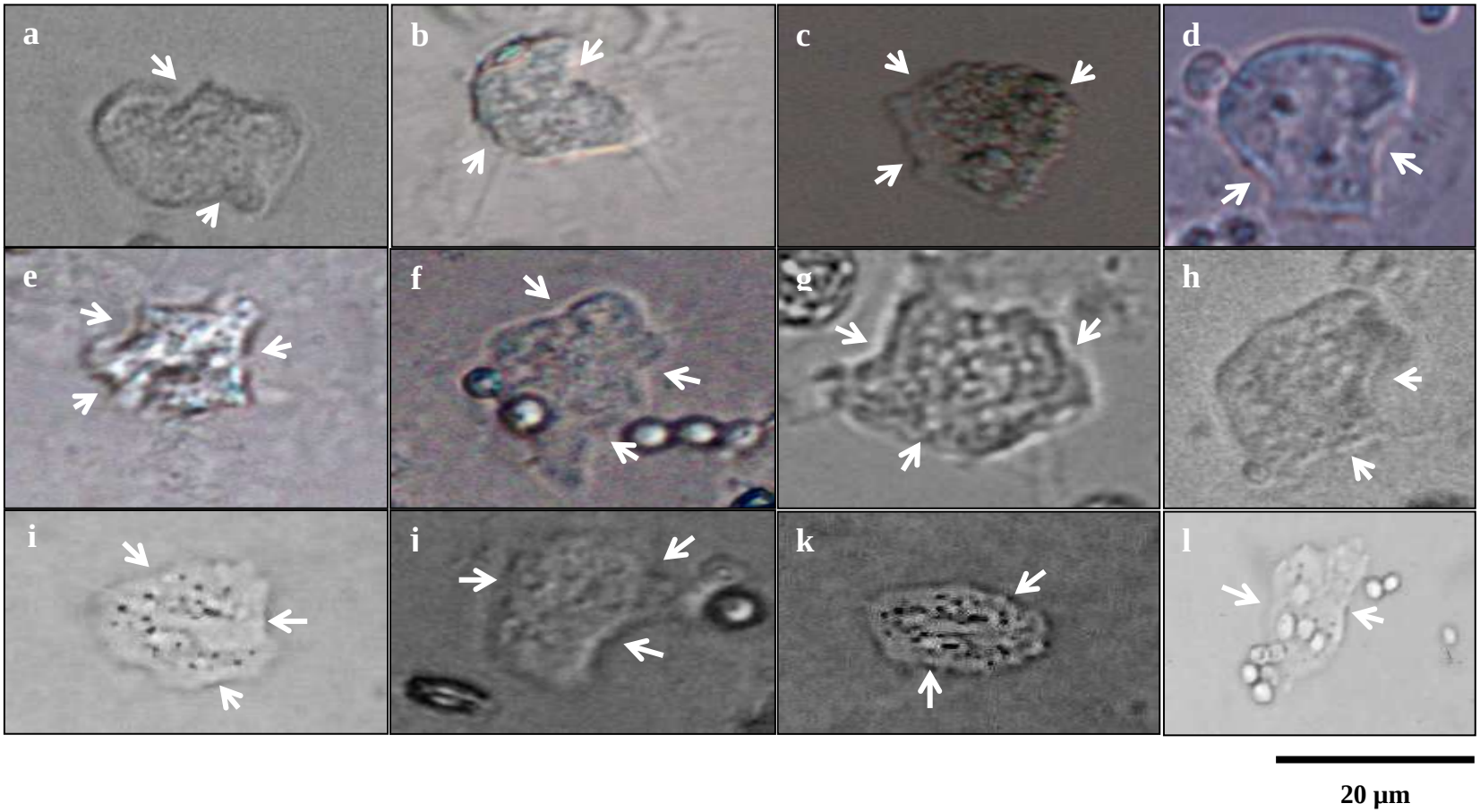


Figure 4.3: Light micrograph showing different types of amoeboid forms of *T. vaginalis* observed on day 3 of suspension culture in cervical neoplasia (CN) isolates; (a-b) CN1, (c-d) CN2, (e-f) CN3, (g-h) CN4, (i-j) CN5, (k-l) CN6.
Note: pseudopodia-like cytoplasmic extensions (*arrows)* with more inclusion-like bodies (black dots) on the cytoplasm of amoeboid forms. Magnifications 400X.

4.3 Growth profiles of trophozoite forms

4.3.1 Growth trend in 10^4 *T. vaginalis*/ml

Generation of growth profiles of trophozoite forms of *T. vaginalis* for nine isolates consisting of three NCN isolates (NCN2 – NCN4) and six CN isolates (CN1 - CN6) with initial inoculum concentration of 1 x 10^4 *T. vaginalis*/ml showed two distinctive and different growth curves (Fig 4.4 and Fig 4.5). The growth profile was generated based on mean count of three replicates (Appendix D). The growth pattern of trophozoites of both CN and NCN isolates started with logarithmic growth phase (log phase) in which the organisms divided continuously until each isolate reached its peak number of parasites. This was then followed by a continuous decrease in the trophozoites count until it reached the death phase where each isolate recorded a zero count of trophozoites.

Presence of motile trophozoite forms were detected in almost all isolates up to 10 days of culture except for CN2 and CN6 which remained viable only up to nine days. Although all the CN isolates were positive for trophozoites form, however there was no significant difference ($p > 0.05$) in average counts between each CN isolates (CN1 – CN6).

All CN isolates exhibited optimum average count of trophozoite forms on day 3 of cultures meanwhile it varied in NCN isolates with NCN2 and NCN3 having peak count on day 4 whilst day 3 for NCN4 isolate (Table 4.3). A gradual increase on the number of trophozoite were observed for all CN isolates and NCN isolates from day 1 to day 3 (NCN4, CN1 – CN6) and day 4 (NCN2 – NCN3) which then decreased greatly upon reaching the optimal count until all parasites become non-viable.

Total average number of trophozoite from day 1 to day 10 counted in CN isolates (CN1 – CN6) and NCN isolates (NCN2 – NCN4) was 3615.04 ± 67.69 x 10^4 *T.*

vaginalis/ml and 1060.63 ± 30.07 x 10^4 *T. vaginalis*/ml respectively. The total average count of trophozoites in NCN isolates was relatively lower than CN isolates. Highest trophozoite count obtained from both types of isolate, CN and NCN was on day 3 with average count of 240 ± 13.89 x 10^4 *T. vaginalis*/ml in CN4 and 92.33 ± 4.93 x 10^4 *T. vaginalis*/ml in NCN4. The lowest trophozoite count recorded was on day 1 for NCN2 with mean count of 5.33 ± 1.53 x 10^4 *T. vaginalis*/ml and day 10 for CN5 with mean count of 0.67 ± 1.15 x 10^4 *T. vaginalis*/ml.

Average trophozoite count on day 3 in all CN isolates were observed to be significantly different ($p < 0.05$) and highest with mean average of 224.22 ± 2.18 x 10^4 *T. vaginalis*/ml compared to other incubation days that at least has one pair that are not significant (Table 4.4). Further data analysis using multiple comparison method (Appendix E) found that mean difference of trophozoite count on day 3 with other days was significant ($p < 0.05$, Dunnett's T3). Based on the growth curve observed for trophozoite in CN isolates (CN1 – CN6), it is evident that the optimal count was on day 3 for all isolates ranging between 210 ± 21.52 x 10^4 *T. vaginalis*/ml for CN2 to 240 ± 13.89 x 10^4 *T. vaginalis*/ml in CN4. This is followed by isolate CN5 with second highest trophozoite count of 238 ± 26.66 x 10^4 *T. vaginalis*/ml. In isolate CN1, there was a rapid increase from day 1 with average count of 16.67 ± 3.06 x 10^4 *T. vaginalis*/ml to day 3 with average count of 221 ± 11.59 x 10^4 *T. vaginalis*/ml. In both types of isolate, trophozoites count started to decrease tremendously after day 7 and none were detected on day 11 of observation.

Table 4.3: The number of trophozoites of *T. vaginalis* in all isolates from day 1 of inoculation up to day 11 where no trophozoites were detected in the cultures.

Isolates	Average no. of trophozoites of *T.vaginalis* x 10^4 *T. vaginalis*/ ml ± standard deviation											
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**
NCN 2	1.00	5.33 ± 1.53	11.00 ± 2.00	41.33 ± 3.21	**89.00 ± 9.17**	86.33 ± 11.85	63.33 ± 5.69	45.33 ± 4.04	19.33 ± 5.86	14.33 ± 6.51	8.00 ± 2.00	0.00
NCN 3	1.00	6.00 ± 1.00	10.00 ± 3.46	50.33 ± 8.74	**87.00 ±9.54**	54.00 ± 5.29	51.33 ±18.23	27.00 ± 3.46	10.67 ± 2.52	5.33 ± 2.08	3.00 ± 2.00	0.00
NCN 4	1.00	13.00 ± 1.73	28.00 ±7.94	**92.3 ± 4.93**	74.33 ±4.04	63.67 ±6.66	46.00 ±10.15	32.33 ±2.52	11.33 ±1.53	8.00 ± 1.00	3.67 ± 1.15	0.00
CN 1	1.00	16.67 ± 3.06	103.00 ± 6.08	**221.00 ± 11.59**	88.00 ± 2.65	64.00 ± 5.29	54.67 ± 3.79	25.33 ± 0.58	15.33 ± 3.51	1.67 ± 2.89	0.33 ± 0.58	0.00
CN 2	1.00	16.00 ± 4.58	99.00 ±8.19	**210.00 ± 21.52**	94.33 ± 8.14	75.00 ± 11.14	24.67 ± 4.51	15.33 ± 3.21	8.67 ± 1.53	3.00 ± 2.65	0.00	0.00
CN 3	1.00	43.67 ± 7.37	85.67 ± 6.66	**209.67 ± 10.26**	149.67 ± 19.04	77.67 ± 3.21	26.33 ± 5.51	20.33 ± 5.51	13.67 ± 4.73	7.33 ± 3.21	2.33 ± 3.21	0.00
CN 4	1.00	17.00 ± 3.46	144.67 ±14.50	**240.00 ± 13.89**	170.33 ± 18.23	68.67 ± 8.62	40.67 ± 4.73	25.00 ± 5.00	12.33 ± 4.16	4. 33 ± 3.21	1.67 ± 2.89	0.00
CN 5	1.00	22.00 ± 6.56	116.00 ± 16.52	**238.00 ± 26.66**	95.00 ± 7.81	58.00 ± 2.65	36.67 ± 5.77	27.33 ± 5.69	16.67 ± 4.93	7.00 ± 3.61	0.67 ± 1.15	0.00
CN 6	1.00	23.67 ± 7.09	67.67 ± 12.86	**226.00 ± 11.36**	92.67 ± 4.73	33.67 ± 2.08	27.00 ± 1.73	17.00 ± 1.00	10.67 ± 0.58	1.67 ± 0.58	0.00	0.00

Note: Values in bold were peak count of trophozoites based on isolates

Values in grey highlighted boxes were significantly different (Dunnett T3).

Table 4.4: Significance analysis between days using Dunnett T3 for average trophozoite count of CN isolatesin 10^4 *T.* vaginalis/ml.

Multiple Comparisons

Dependent Variable: Average trophozoites count in 10^4 *T. vaginalis*/ml

Dunnett T3

(I) Days of count	(J) Days of count	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Day 3	Day 0	223.22333*	5.36184	**.000**	193.6285	252.8181
	Day 1	201.05500*	6.87041	**.000**	171.0052	231.1048
	Day 2	121.55500*	12.01630	**.000**	64.7365	178.3735
	Day 4	109.22333*	15.47124	**.008**	32.1207	186.3260
	Day 5	161.38833*	8.44575	**.000**	124.5406	198.2361
	Day 6	189.22167*	7.14190	**.000**	158.2293	220.2140
	Day 7	202.50333*	5.72359	**.000**	174.0276	230.9791
	Day 8	211.33333*	5.49660	**.000**	182.3122	240.3545
	Day 9	220.05667*	5.46006	**.000**	190.9010	249.2123
	Day 10	223.39000*	5.37614	**.000**	193.8667	252.9133

*. The mean difference is significant at the 0.05 level

Note: Comparison of average trophozoite count on day 3 with the rest of the days yielded all positive values with p-value less than 0.05 (bold). The 95 % confidence interval does not contain the value 0. Thus, there is a significant difference of average trophozoite count between days with day 3 having significantly higher count.

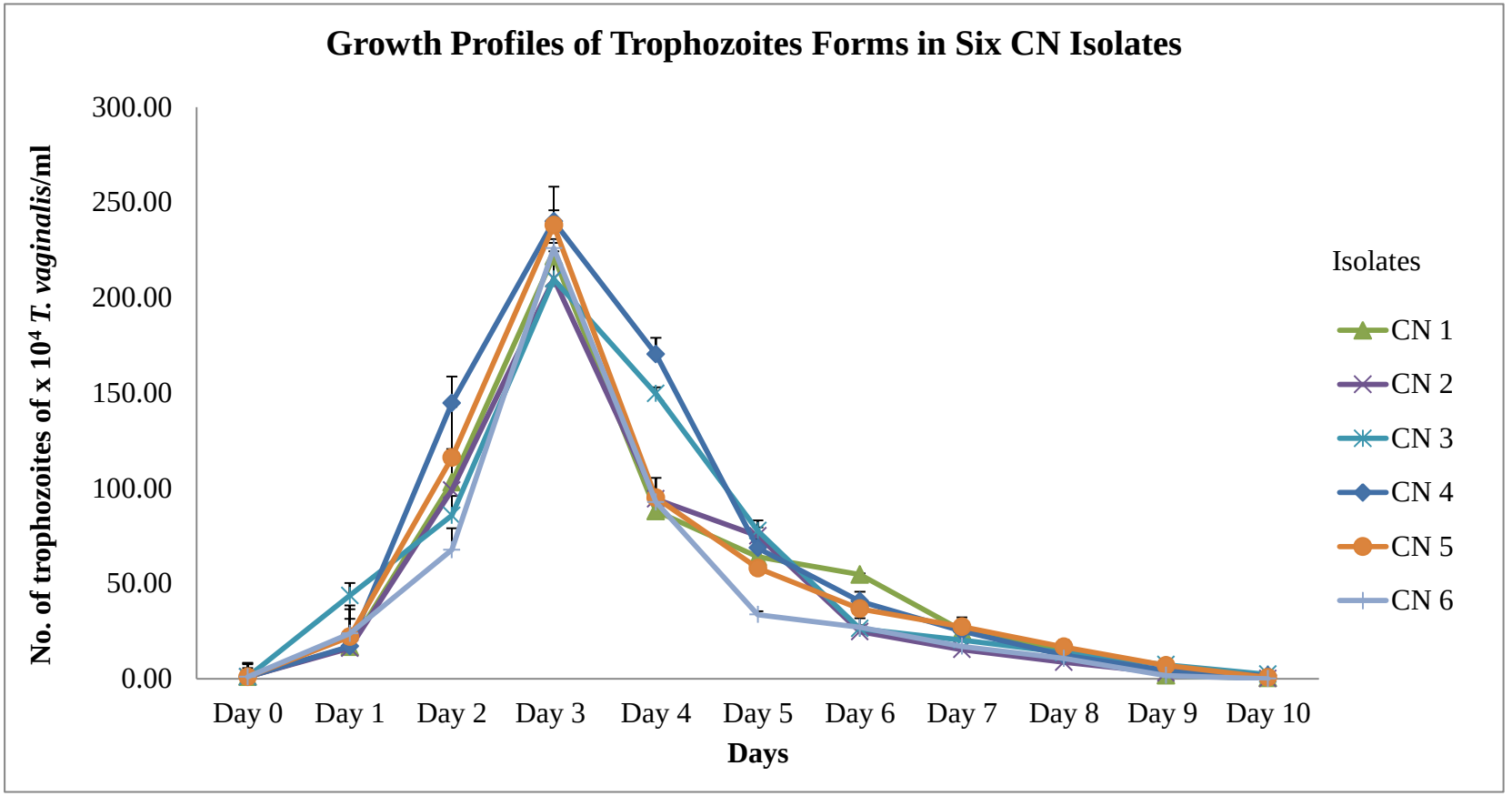


Figure 4.4: Growth profiles of trophozoite forms in CN isolates (Isolates 1 – Isolates 6) at 10^4 *T. vaginalis*/ml inoculated in Hollander medium containing 10 % heat inactivated horse serum and 2 % PSN antibiotic mixture and incubated at 37ºC. All CN isolates exhibited peak count of trophozoite forms on day 3 culture.

Note: The values were expressed as the means ± S.D.

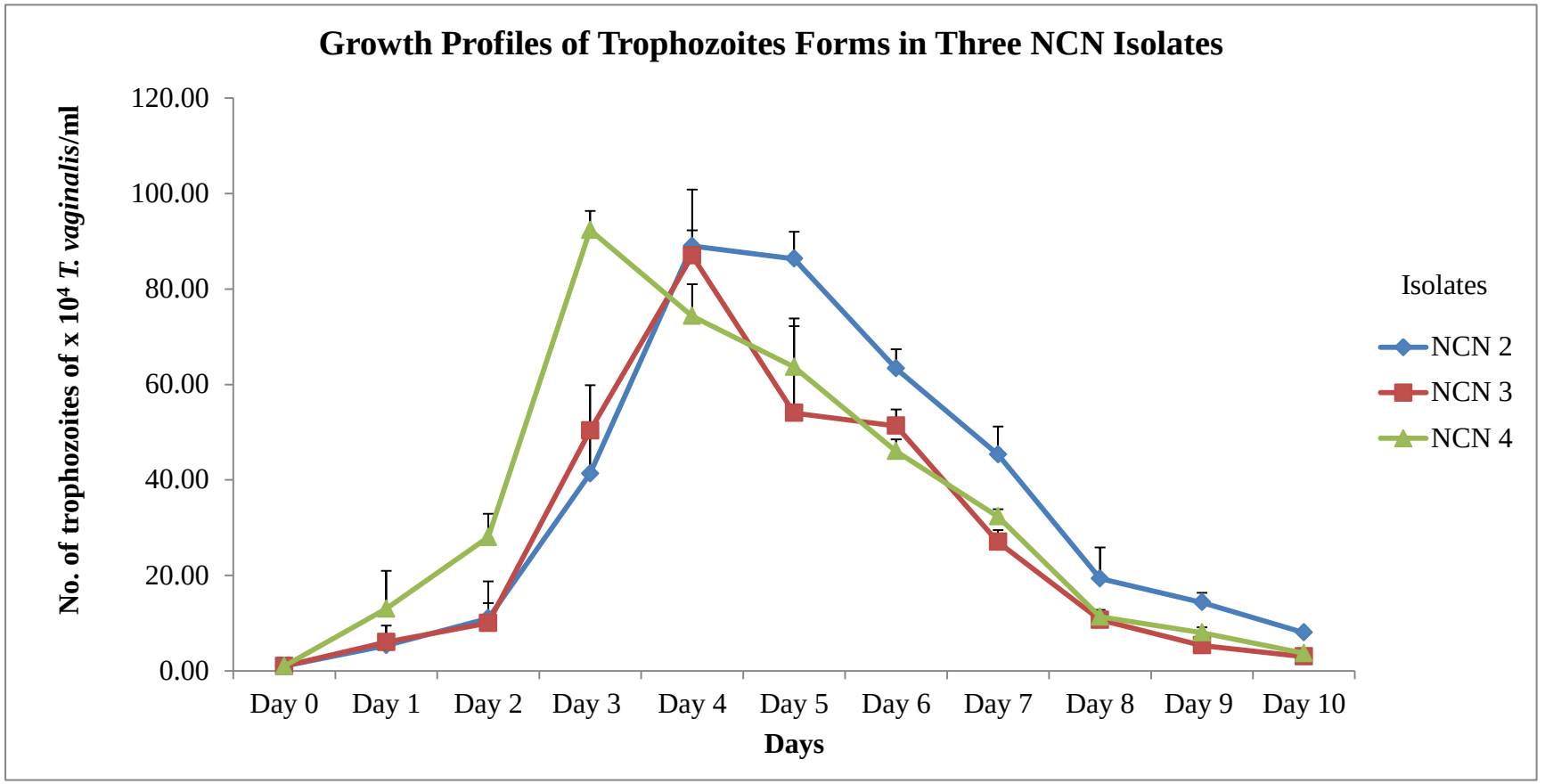


Figure 4.5: Growth profiles of trophozoite forms in NCN isolates (Isolates 2 – Isolates 4) at 10^4 *T. vaginalis*/ml inoculated in Hollander medium containing 10% heat inactivated horse serum and 2% PSN antibiotic mixture and incubated at 37ºC. NCN4 showed peak count on day 3 meanwhile NCN2 and NCN3 showed peak count on day 4.
Note: The values were expressed as the means ± S.D.

4.3.2 Growth trends in 10^5 *T. vaginalis*/ml

Growth profile of average trophozoite counts of *T. vaginalis* for CN isolates in 10^5 *T. vaginalis*/ml showed a similar curve with 10^4 *T. vaginalis*/ml but with increased trophozoite counts (Table 4.5 and Fig 4.6). The growth profile generated was based on mean count of three replicates (Appendix F). There was no significant difference in mean trophozoite counts between each CN isolate tested as well as between the days ($p > 0.05$). However, the average trophozoites count was the highest on day 3 with mean total of 429.22 ± 11.19 x 10^5 *T. vaginalis*/ml. In all CN isolates, the trophozoites were observed to be viable only for six days. Among all the CN isolates, CN6 produced the most trophozoite forms with the count of 512.67 ± 12.74 x 10^5 *T. vaginalis*/ml on day 3 cultures. There was a rapid growth of trophozoites from day 1 to day 3 and decreased tremendously thereafter.

Table 4.5: The average number of trophozoites of *T. vaginalis* in all CN isolates at inoculum concentrations of 10^5 *T. vaginalis*/ml.

Isolates	**Average count (No of trophozoites x 10^5 *T. vaginalis*/ ml ± S.D.**							
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
CN 1	1.00	62.67 ±11.02	121.00 ± 14.53	**361.67 ± 32.52**	237.00 ± 8.00	65.33 ± 18.50	3.00 ± 1.00	0.00
CN 2	1.00	31.67 ±11.02	150.00 ±19.7	**370.33 ± 43.02**	221.33 ±21.01	34.00 ± 14.11	6.33 ± 3.21	0.00
CN 3	1.00	41.33 ± 4.04	139.67 ± 23.97	**510.33 ± 63.14**	267.33 ± 19.04	41.00 ± 3.61	13.67 ± 6.43	0.00
CN 4	1.00	78.67 ± 9.61	141.67 ± 8.08	**421.33 ± 51.64**	227.67 ± 21.08	91.00 ± 13.89	23.67 ±10.07	0.00
CN 5	1.00	35.00 ± 4.58	167.67 ± 14.47	**399.00 ± 19.00**	218.00 ± 16.82	53.33 ± 21.73	0.33 ± 0.58	0.00
CN 6	1.00	51.00 ±17.09	127.33 ± 26.16	**512.67 ± 12.74**	230.67 ± 28.01	109.67 ± 12.06	33.33 ± 9.07	0.00

Note: Values highlighted in grey were highest for trophozoites count.

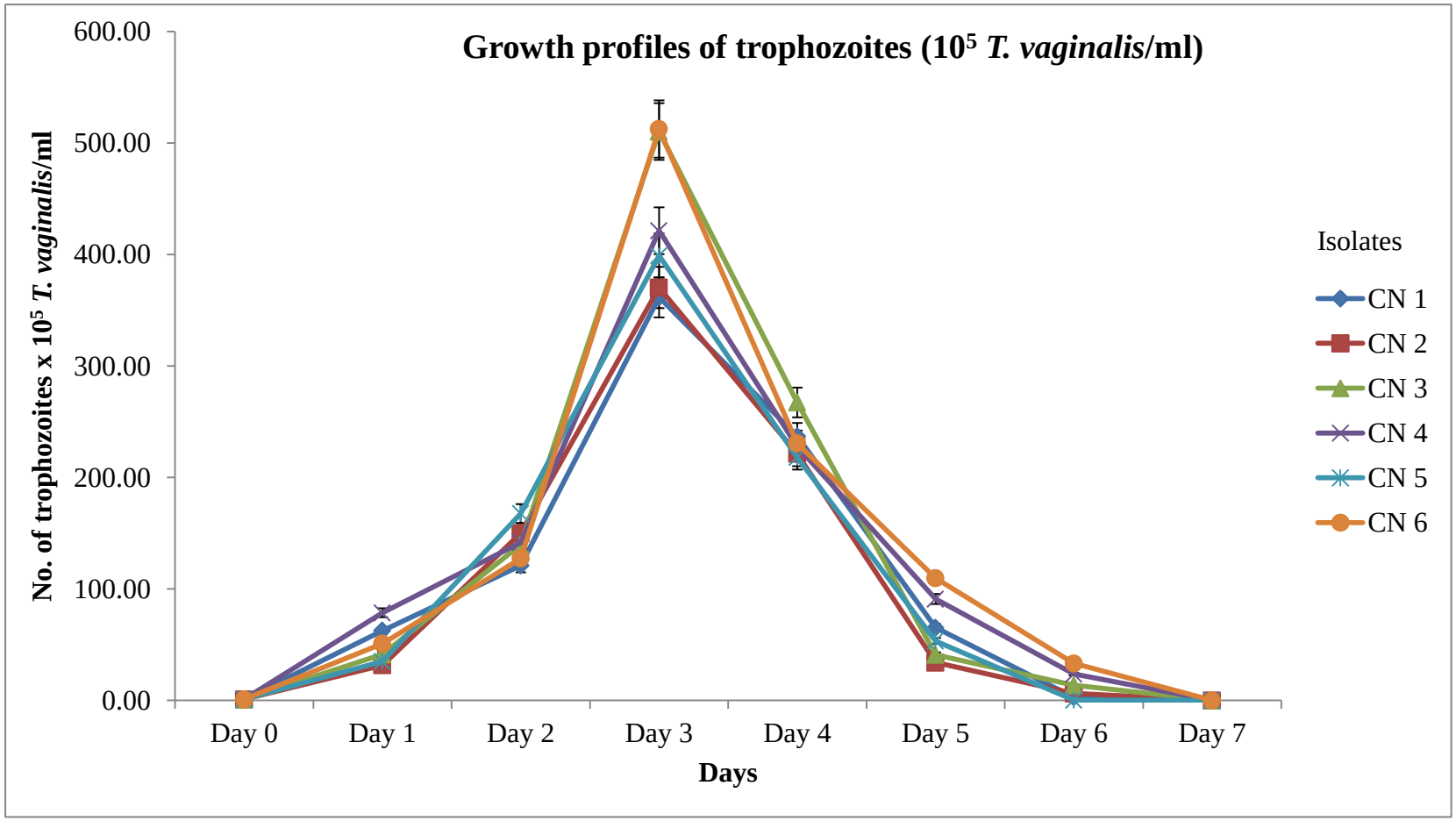


Figure 4.6: Growth profiles of trophozoite forms in CN isolates (Isolates 1 – Isolates 6) at 10^5 *T. vaginalis*/ml inoculated in Hollander medium containing 10 % heat inactivated horse serum and 2 % PSN antibiotic mixture and incubated at 37ºC. All the CN isolates showed peak count on day 3 culture.

Note: The values were expressed as the means ± S.D.

4.3.3 Combined growth profiles of CN isolates for 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml

The combined average growth rate of trophozoites in all CN isolates in 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml were shown in Table 4.6. The average growth rate of trophozoites from day 1 to day 4 was significantly higher ($p < 0.05$) when parasite concentration was 10^5 *T. vaginalis*/ml and the growth was overtaken by parasite concentration of 10^4 *T. vaginalis*/ml from day 6 onwards (Table 4.7). Trophozoite count was approximately the same on day 5, thus no significant difference were produced between the two concentrations.

The mean average trophozoite form observed in all CN isolates (CN1-CN6) was 597.51 ± 8.93 x 10^4 *T. vaginalis*/ml for 10^4 *T. vaginalis*/ml and 933.28 ± 18.71 x 10^5 *T. vaginalis*/ml for 10^5 *T. vaginalis*/ml. The trophozoite count was constantly higher in 10^5 *T. vaginalis*/ml from day 1 to day 4 of culture and decreased steadily from day 5 until all the parasites attained senescence. Based on the graph (Fig. 4.7), it can be observed that trophozoite forms became totally non-viable first in culture with concentration of 10^5 *T. vaginalis*/ml on day 7 followed by trophozoites in 10^4 *T. vaginalis*/ml on day 10.

Table 4.6: The average growth of trophozoites in all CN isolates (CN1-CN6) at10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml.

Isolates	**Total Average count (No of trophozoites x 10^x *T. vaginalis*/ ml ± S.D.**								
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
10^4 *T. vaginalis*/ml	1.00	23.17 ± 1.75	102.67 ± 4.39	224.22 ± 2.18	115.00 ± 5.92	62.84 ± 2.66	35.00 ± 1.93	21.72 ± 0.82	12.89 ±0.82
10^5 *T. vaginalis*/ ml	1.00	50.06 ± 3.00	141.22 ± 2.77	429.22 ± 11.19	233.67 ± 2.97	65.72 ± 4.92	13.39 ± 2.15	0.00	0.00

Note: ^{x} represents the concentration of the parasites.

Table 4.7: Significant difference of growth rates of trophozoites in CN isolates between inoculum size of 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml using Mann-Whitney Test.

	Test Statistics[a]							
	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
Mann-Whitney U	3.000	4.000	.000	.000	18.000	3.000	.000	.000
Wilcoxon W	24.000	25.000	21.000	21.000	39.000	24.000	21.000	21.000
Z	-2.402	-2.242	-2.882	-2.882	.000	-2.402	-3.077	-3.077
Asymp. Sig. (2-tailed)	.016	.025	.004	.004	1.000	.016	.002	.002
Exact Sig. [2*(1-tailed Sig.)]	.015[b]	.026[b]	.002[b]	.002[b]	1.000[b]	.015[b]	.002[b]	.002[b]
	**	**	**	**		***	***	***

a. Grouping Variable: Parasites concentrations

b. Not corrected for ties.

Note: Comparison of growth rates of trophozoites in CN isolates (CN1-CN6) between parasite concentration of 10^4 *T.* vaginalis/ml and 10^5 *T. vaginalis*/ml. Trophozoites count were significantly higher from day 1 to day 4 in 10^5 *T. vaginalis*/ml (** $p < 0.005$, Mann-Whitney Test) and from day 6 to day 8 in 10^4 *T. vaginalis*/ml (*** $p < 0.005$, Mann-Whitney Test).

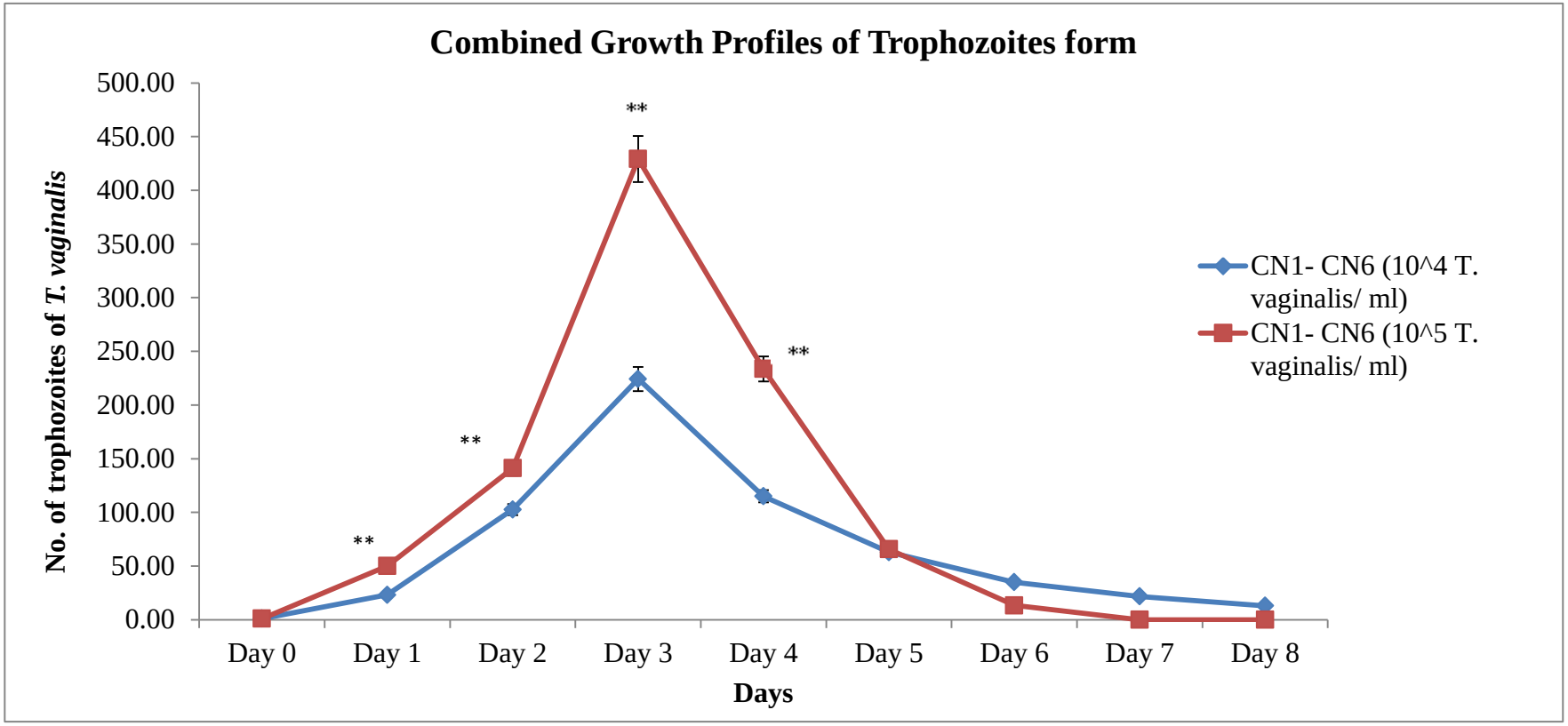


Figure 4.7: Comparison of combined growth profiles of average trophozoite forms of *T. vaginalis* between 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml in all CN isolates (CN1-CN6). Total average trophozoite form found in all CN isolates of 10^5 *T. vaginalis*/ml from day 1 to day 4 culture was significantly higher than in 10^4 *T. vaginalis*/ml (** $p < 0.05$, Mann-Whitney Test).

Note: The values were expressed as mean average ± S.D.

4.4 Assessment of amoeboid morphology by staining method

Once the amoeboid forms were detected using light microscopy and further confirmed with growth profile study, the positive isolates were subjected to staining methods to detect and analyze the morphological characteristics of the amoeboid forms. In this study, the positive isolates were CN isolates (CN1-CN6). The two types of staining methods used were the Giemsa stain and the Modified Field's Stain.

Nucleus and cytoplasm of the amoeboid forms of *T. vaginalis* were stained dark purple and light purple respectively with Giemsa stain. Meanwhile with Modified Field's Stain the nucleus and cytoplasm were stained with bright purplish red and light purplish red respectively. Inactive flagella although was observed attached to the cytoplasm of amoeboid forms did not pick up the stains by both methods. Morphological differences between trophozoite and amoeboid forms of *T. vaginalis* were highlighted through the staining methods (Fig 4.8a & Fig. 4.8b). Giemsa stained the flagella of trophozoites light purple while Field's stain showed reddish purple. This was absent in the amoeboid forms.

Despite the significant colour contrast of amoeboid forms observed using both the stains, staining with Field's stain was found to be more rapid (15 seconds) compared to 30 minutes for the Giemsa stain. Field's stain was better than Giemsa stain as it gave better and noticeable contrast of the amoeboid morphological features. The nucleus, cytoplasm and the pseudopodia-like projections were more clearly seen in Field's stain. Other morphological features such as single or multi-nucleus as well rough surfaces were clearer when stained with Field's stain (Figure 4.9).

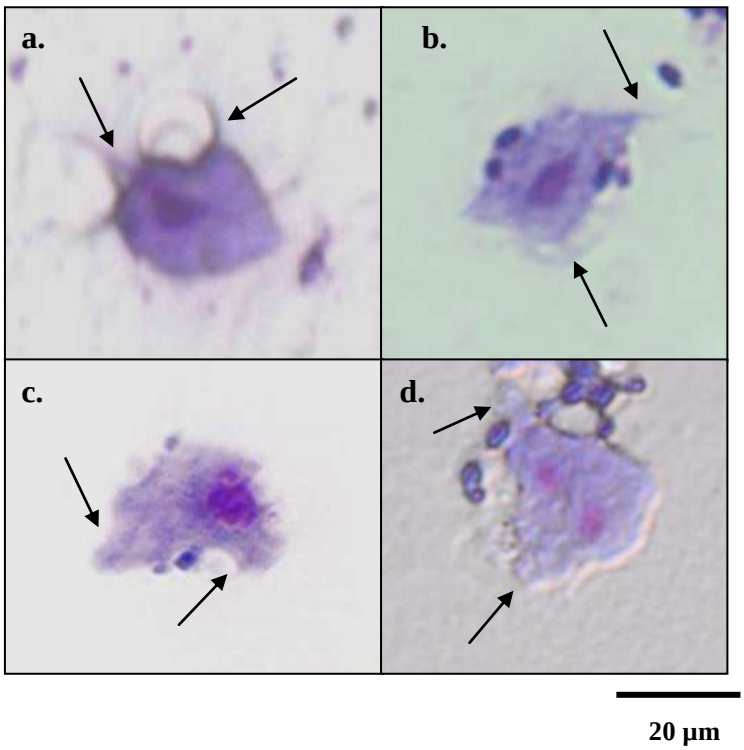


Figure 4.8a: Morphological comparison between Giemsa stained (a-b) trophozoites and (c-d) amoeboid forms. Note the evident differences between ovoid trophozoites and abnormal amoeboid. The arrows indicate the flagella (a-b) and pseudopodia-like cytoplasmic projections (c-d). Magnifications 400X.

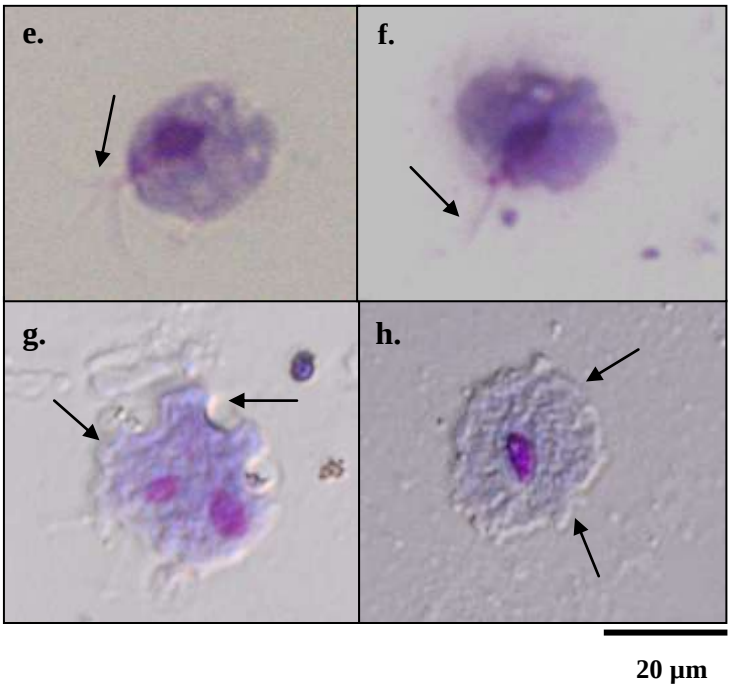


Figure 4.8b: Morphological comparison between Modified Field's stained (e-f) trophozoites and (g-h) amoeboid forms. Note the evident differences between ovoid trophozoites and abnormal amoeboid. The arrows indicate the flagella (e-f) and pseudopodia-like cytoplasmic projections (g-h). Magnifications 400X.

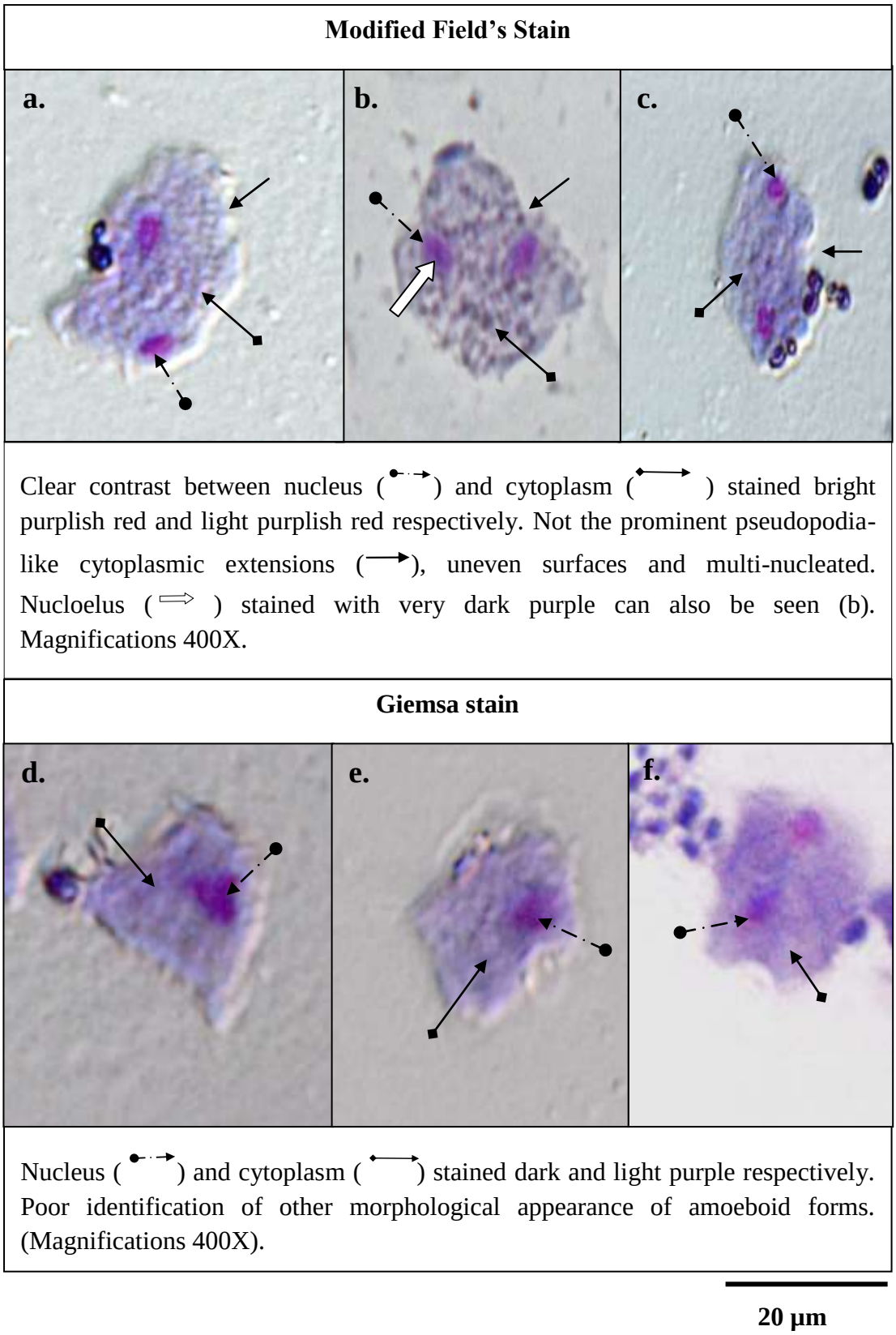


Figure 4.9: Comparison of staining method between (a-c) Modified Field's Stain and (d-f) Giemsa stain of amoeboid forms in CN isolates of *T. vaginalis*.

4.5 Effect of different stress conditions in triggering amoeboid forms

Based on the three different stress conditions applied to the suspension culture, formation of amoeboid forms in *T. vaginalis* was observed to be significantly higher ($p < 0.05$) when the growth conditions were stressed than in normal conditions in all the CN isolates (CN1 – CN6). Although the mean counts of amoeboid forms were different in each CN isolate for the three respective stress conditions, yet there was no significant relationship ($p > 0.05$) between each isolate in triggering this form. Raw data on the average count of the amoeboid forms for the three stress conditions tested are shown in Appendix G – Appendix I.

4.5.1 Effect of different sizes of inoculum

Among the three different types of *T. vaginalis* concentrations compared, amoeboid forms of *T. vaginalis* were only seen in culture with inoculum size of 10^5 *T. vaginalis*/ml. No amoeboid forms were present in culture with 10^3 *T. vaginalis*/ml and 10^6 *T. vaginalis*/ml. Appearance of amoeboid forms were found to be similar with the growth profile of amoeboid form at 10^4 *T. vaginalis*/ml but with increased number of trophozoites and amoeboid forms were detected on day 1 itself for all the CN isolates (Table 4.8 and Fig. 4.10). All CN isolates (CN1 – CN6) reported significantly higher number of amoeboid forms ($p < 0.05$) from day 1 to day 4 when the number of *T. vaginalis* was increased ten-fold from the standard concentration of 10^4 *T. vaginalis*/ml (Table 4.9 and Fig. 4.11). Data on statistical significance between average amoeboid counts between 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml were shown in Appendix J.

Total number of amoeboid forms detected in culture of 10^5 *T. vaginalis*/ml was $116.65 \pm 3.96 \times 10^5$ *T. vaginalis*/ml with peak count of $59.33 \pm 3.03 \times 10^5$ *T. vaginalis*/ml on day 3 of culture in all CN isolates (CN1-CN6). Lowest amount of amoeboid forms were detected on day 4 with total count of $7.67 \pm 0.53 \times 10^5$ *T.*

vaginalis/ml. Despite statistical insignificance between CN isolates, isolate CN4 recorded highest numbers of amoeboid forms with mean average of 4.61 ± 0.91 x 10^5 *T. vaginalis*/ml. Only mean average of 2.22 ± 0.46 x 10^5 *T. vaginalis*/ml of amoeboid were detected in isolate CN1. Overall, all the CN isolates developed amoeboid forms only up to 96 hours of incubation.

Table 4.8: Average amoeboid forms of *T. vaginalis* in CN isolates from day 1 to day 5 in 10^5 *T. vaginalis*/ml.

Isolates	**Average count (No of amoeboid x 10^5 *T. vaginalis*/ ml ± S.D)**					
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
CN 1	0.00	1.33 ± 0.58	3.00 ± 0.00	**7.33 ± 3.06**	1.67 ± 0. 58	0.00
CN 2	0.00	2.00 ± 1.73	5.33 ± 0.58	**14.33 ± 4.93**	1.33 ± 0.58	0.00
CN 3	0.00	1.33 ± 0.58	7.00 ± 1.00	**7.67 ± 2.52**	0.33 ± 0.58	0.00
CN 4	0.00	3.33 ± 1.53	9.67 ± 1.53	**13.00 ± 3.46**	1.67 ± 1.53	0.00
CN 5	0.00	3.00 ± 1.73	7.33 ± 3.79	**9.33 ± 3.79**	1.67 ± 1.15	0.00
CN 6	0.00	2.00 ± 0.00	4.33 ± 1.15	**7.67 ± 1.15**	1.00 ± 1.00	0.00

Note: Values in bold and highlighted were peak count of amoeboid forms on day 3.

Table 4.9: The significant difference between growth profiles of amoeboid forms of at 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml using Mann-Whitney test.

	Test Statistics[a]			
	Day 1	Day 2	Day 3	Day 4
Mann-Whitney U	.000	.000	.000	4.500
Wilcoxon W	21.000	21.000	21.000	25.500
Z	-2.950	-2.908	-2.898	-2.263
Asymp. Sig. (2-tailed)	.003	.004	.004	.024
Exact Sig. [2*(1-tailed Sig.)]	.002[b]	.002[b]	.002[b]	.026[b]
	**	**	**	**

a. Grouping Variable: Cell concentrations b. Not corrected for ties.

Note: Comparison of growth rates of amoeboid forms in CN isolates (CN1-CN6) between 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml. Amoeboid forms were significantly higher from day 1 to day 4 in 10^5 *T. vaginalis*/ml (** $p < 0.05$, Mann-Whitney Test).

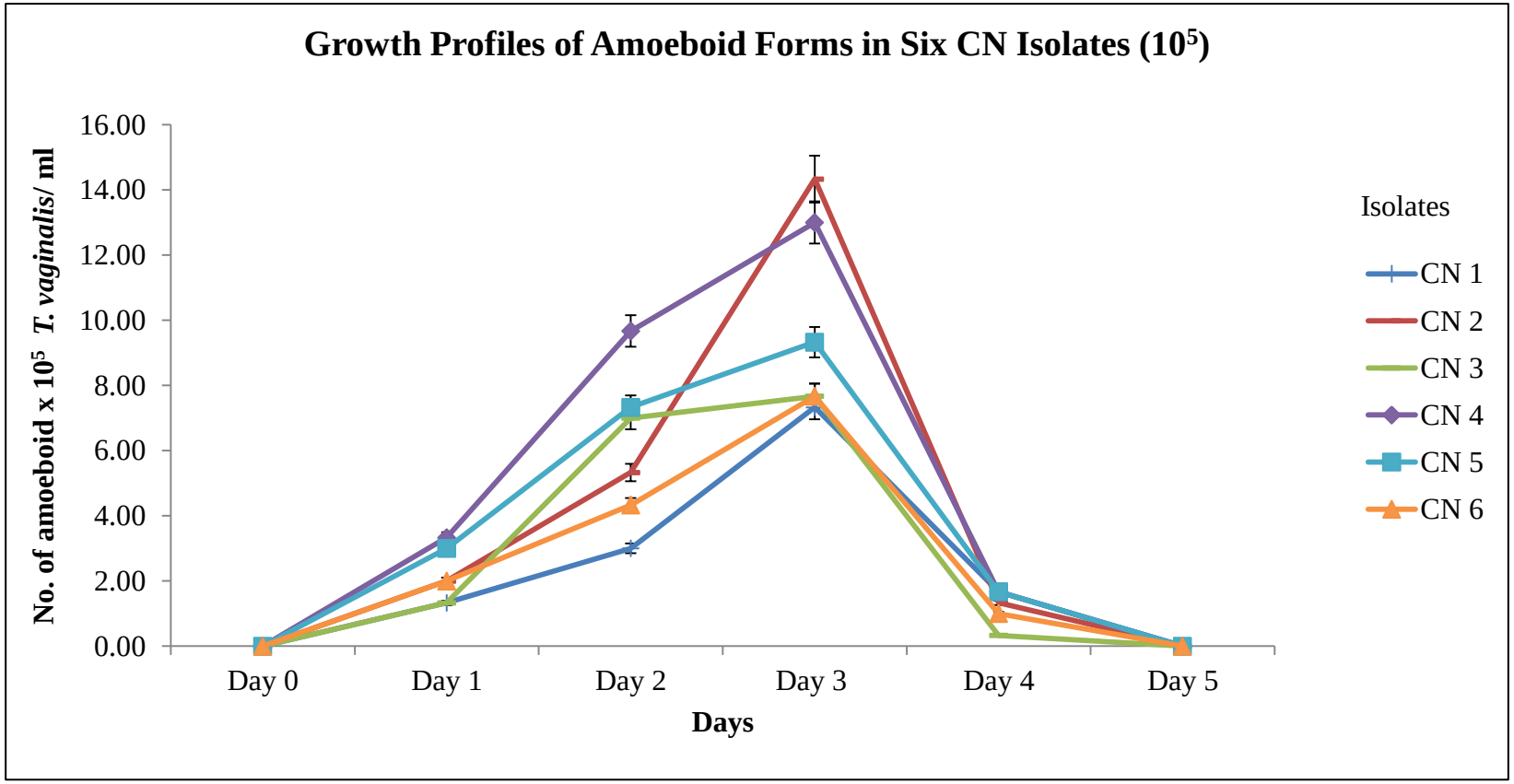


Figure 4.10: Growth profiles of amoeboid forms of *T. vaginalis* in CN isolates (CN1 – CN6) at 10^5 *T. vaginalis*/ml inoculated in Hollander medium supplemented with 10 % Horse serum and 2 % Penicillin-Streptomycin Neomycin antibiotic mixture with optimum growth on day 3 of culture for all CN isolates.

Note: The values were expressed as average ± S.D.

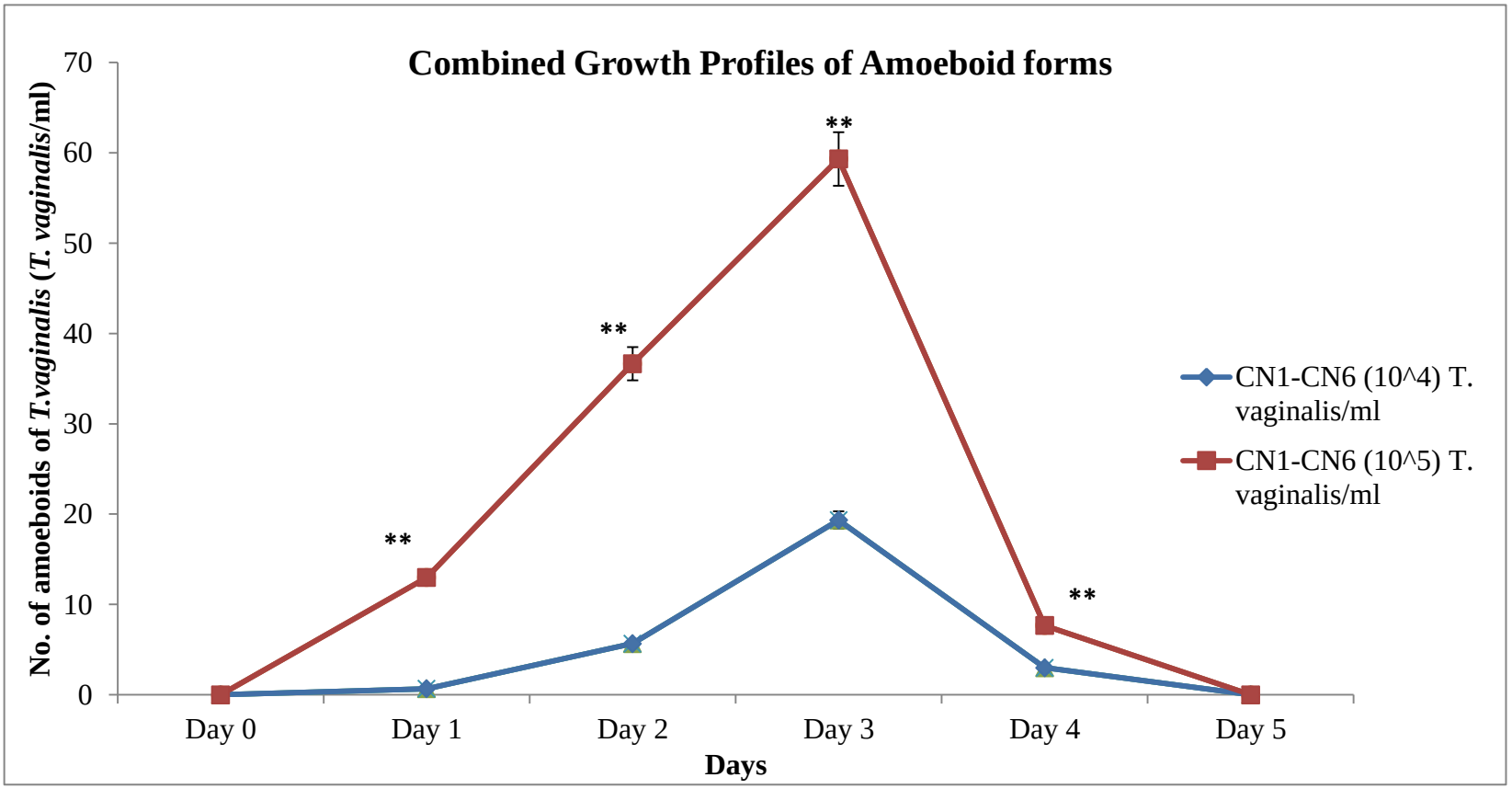


Figure 4.11: Comparison of combined growth profiles of average amoeboid forms of *T. vaginalis* between inoculum size of 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml in all CN isolates (CN1-CN6). Total average amoeboid found in all CN isolates of 10^5 *T. vaginalis*/ml from day 1 to day 4 culture was significantly higher than in 10^4 *T. vaginalis*/ml (** $p < 0.05$, Mann-Whitney Test). Note: The values were expressed as mean average ± S.D.

4.5.2 Effect of different concentrations of horse serum

All six isolates of CN responded well to different concentrations of horse serum ranging from the lowest concentration of 1 % to highest concentration of 20 % (Table 4.10). Horse serum concentrations of 10 % were used as the reference standard to evaluate the formation of amoeboid forms of *T. vaginalis.* Percentages of amoeboid forms in CN isolates of *T. vaginalis* were observed to vary greatly when the concentrations of horse serum were increased from 1 % to 20 % (Fig.4.12). Amoeboid forms were not observed in any of CN isolates when the horse serum concentrations were the lowest (1 %), thus reported a significantly lowest count ($p < 0.05$) compared to other concentrations. 15 % of horse serum triggered the highest formation of amoeboid in all CN isolates with mean average of 9.95 ± 0.47 x 10^4 *T. vaginalis*/ml and was significantly higher ($p < 0.05$) than the amoeboid forms identified in horse serum concentrations of 1 %, 5 % and 10 %. When the concentration was further increased to 20 %, amoeboid forms were still observed in the culture, however the count decreased slightly to mean average of 6.33 ± 0.27 x 10^4 *T. vaginalis*/ml with no significant difference with 5 %, 10 % and 15 % concentrations. Data on statistical significance were presented in Appendix K.

Table 4.10: Average amoeboid forms between different horse serum concentrations in CN isolates.

Isolates	**Average amoeboid forms ± S.D x 10^4 *T. vaginalis*/ml**				
	1 %	**5 %**	**10 %**	**15 %**	**20 %**
CN 1	0.00	4.00 ± 1.26	5.00 ± 1.37	**11.00 ± 2.49**	5.67 ± 1.59
CN 2	0.00	3.00 ± 0.83	4.33 ± 1.19	**7.33 ± 1.80**	8.00 ± 2.39
CN 3	0.00	3.67 ± 0.98	3.67 ± 1.06	**7.33 ± 2.14**	6.33 ± 1.77
CN 4	0.00	3.00 ± 0.89	3.00 ± 0.72	**8.67 ± 2.23**	5.67 ± 1.66
CN 5	0.00	4.00 ± 1.26	4.00 ± 1.03	**14.67 ± 4.02**	8.33 ± 2.29
CN 6	0.00	6.00 ± 1.79	6.00 ± 1.16	**10.67 ± 3.35**	4.00 ± 1.10

Note: Horse serum concentrations of 15 % reported highest count of amoeboid forms (bold and highlighted).

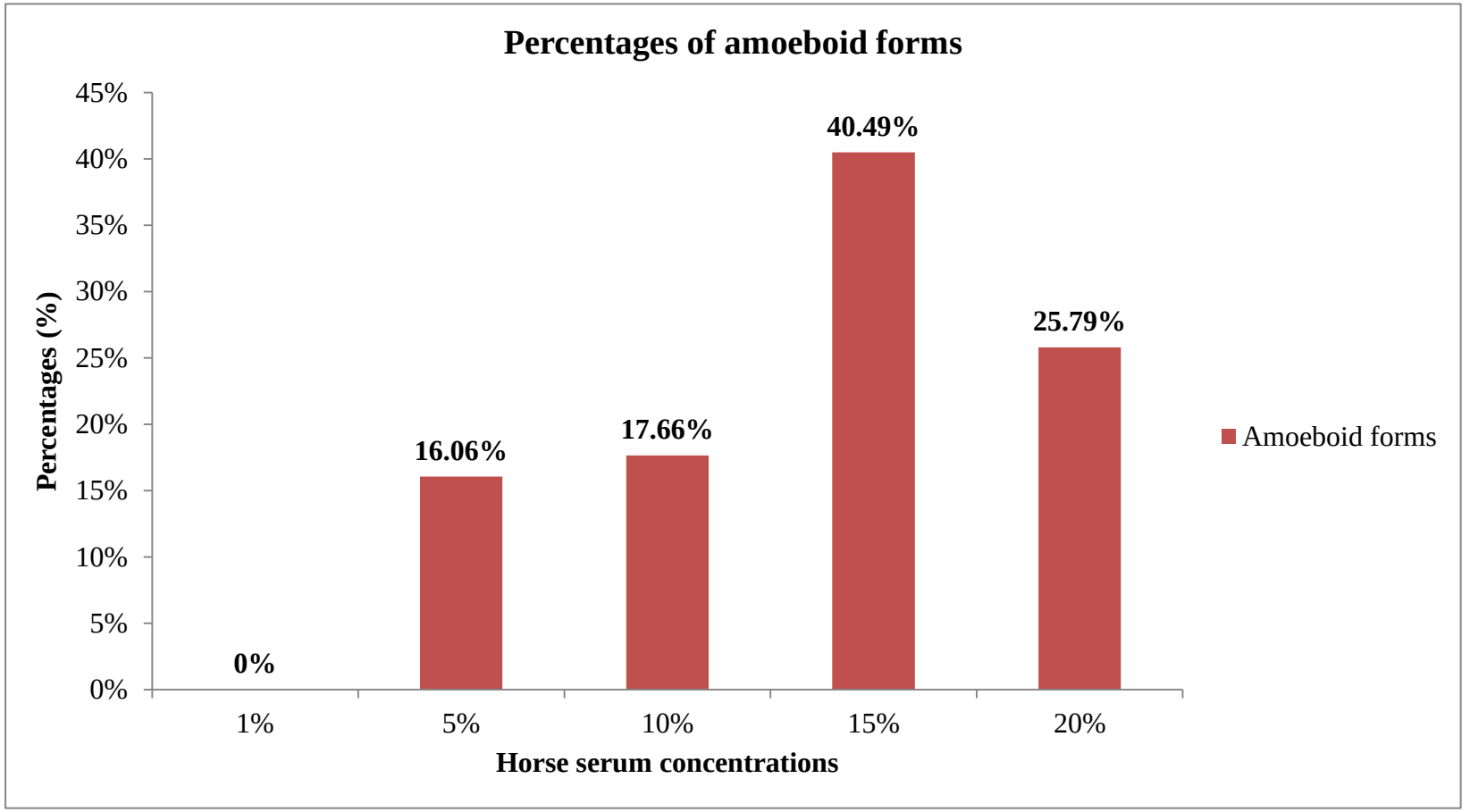


Figure 4.12: Effect of different horse serum concentrations in triggering amoeboid forms in CN isolates (CN1 – CN6) grown in Hollander medium.

Note: Horse serum concentration of 15 % reported higher count of amoeboid in all CN isolates while none in 1 %.

4.5.3 Effect of different concentrations of metronidazole drug

In the final evaluation of the effects of growth environment on the development of amoeboid forms, the culture conditions of CN isolates was put under stress by introducing different concentrations of metronidazole drug. Effects of metronidazole drug was observed to trigger amoeboid forms under certain circumstances (Fig.4.13) and CN isolates were found to be susceptible to high concentrations of the drug. Among the five different metronidazole drug concentrations tested, amoeboid forms were only observed at the two lowest concentrations of 0.001 mg/ml and 0.0001 mg/ml with significantly higher count in 0.0001 mg/ml ($p < 0.05$). The mean average of amoeboid forms detected in 0.001 mg/ml and 0.0001 mg/ml was $1.56 \pm 0.08 \times 10^4$ *T. vaginalis*/ml and $3.61 \pm 0.07 \times 10^4$ *T. vaginalis*/ml respectively. However, when compared with the control (without metronidazole drug), the amoeboid forms in all the six CN isolates were seen to be significantly higher ($p < 0.05$) using Tukey HSD compared to 0.001 mg/ml and 0.0001 mg/ml with mean average amoeboid forms of $5.66 \pm 0.13 \times 10^4$ *T. vaginalis*/ml (Table 4.11). Data on statistical analysis were presented in Appendix L.

Although there was no significant preference between CN isolates in developing amoeboid forms, highest amoeboid forms in control and 0.0001 mg/ml was in isolate CN2 meanwhile in 0.001 mg/ml was in isolate CN3.

Table 4.11: Comparison of average amoeboid forms in CN isolates between control and different concentrations of metronidazole drug.

Isolates	**Average amoeboid forms ± S.D x 10^4 *T. vaginalis*/ml**		
	Control	**0.001 mg/ml**	**0.0001 mg/ml**
CN 1	6.00 ± 1.30	1.67 ± 0.26	3.33 ± 0.26
CN 2	7.00 ± 1.52	1.67 ± 0.26	4.33 ± 0.46
CN 3	5.00 ± 1.00	2.33 ± 0.26	3.67 ± 0.67
CN 4	5.00 ± 1.41	1.67 ± 0.26	3.33 ± 0.52
CN 5	5.00 ± 1.41	1.00 ± 0.45	3.67 ± 0.26
CN 6	6.00 ± 1.30	1.00 ± 0.00	3.33 ± 0.87

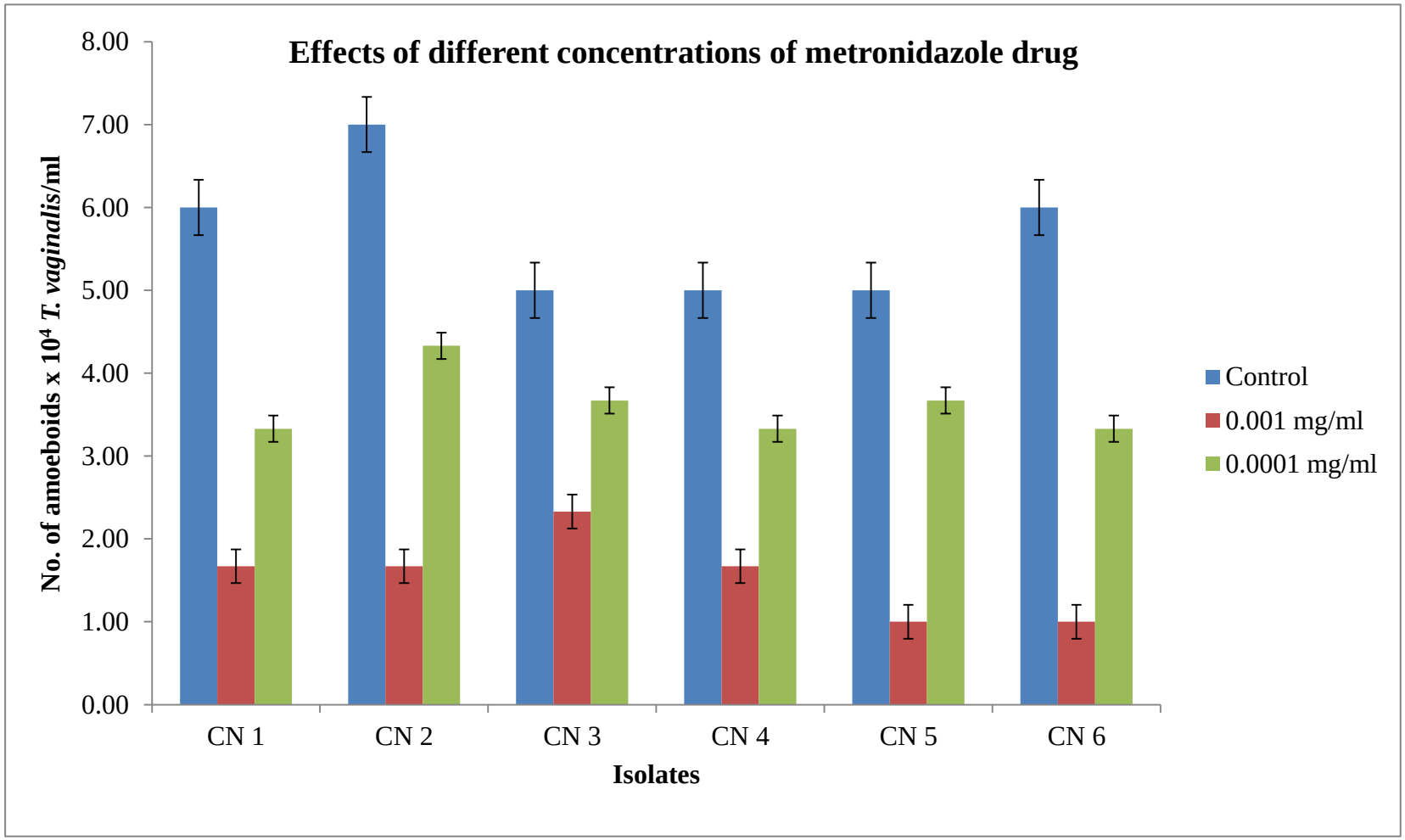


Figure 4.13: Effects of different concentrations of metronidazole drug against control in CN isolates (CN1-CN6). Highest amoeboid forms were observed in conditions without metronidazole drug followed by in 0.0001 mg/ml.
Note: Values were expressed as mean ± S.D.
Standard bar = Standard error.

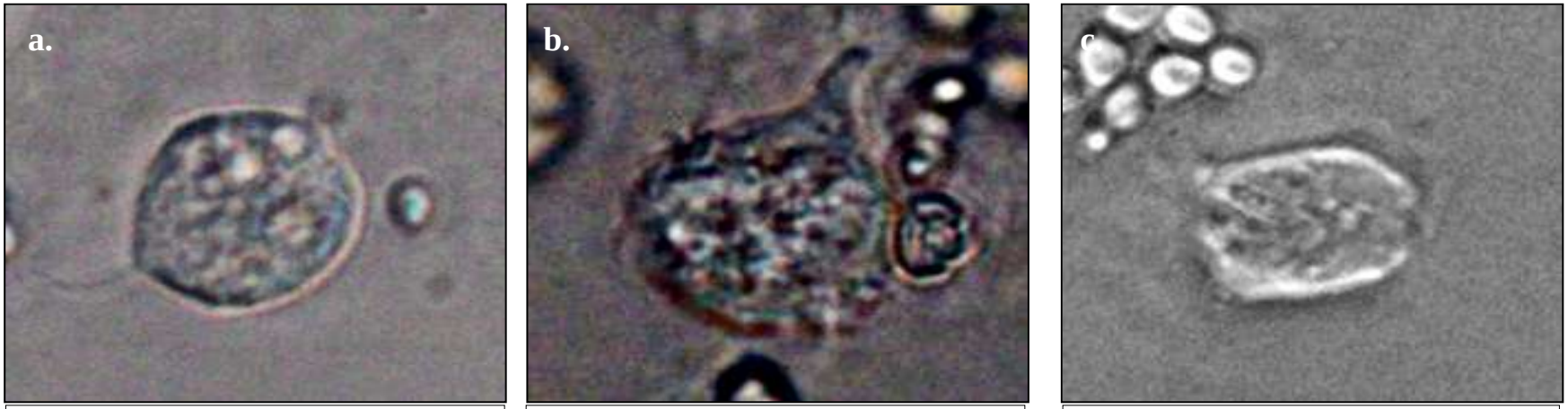
4.6 Transformational changes analysis from trophozoites to amoeboid forms

Transformational changes in CN isolates of *T. vaginalis* were successfully demonstrated using light microscopy. There were significant modifications of the ovoid trophozoite forms when changing into irregular amoeboid forms. The changes were observed under two types of conditions namely stress-free and stressed culture environments. Comparison of the morphological changes from trophozoites to amoeboid forms between these two conditions revealed remarkable variations to the size and shapes of CN isolates with evident observations in stressed culture conditions (Fig 4.14 & Fig 4.15).

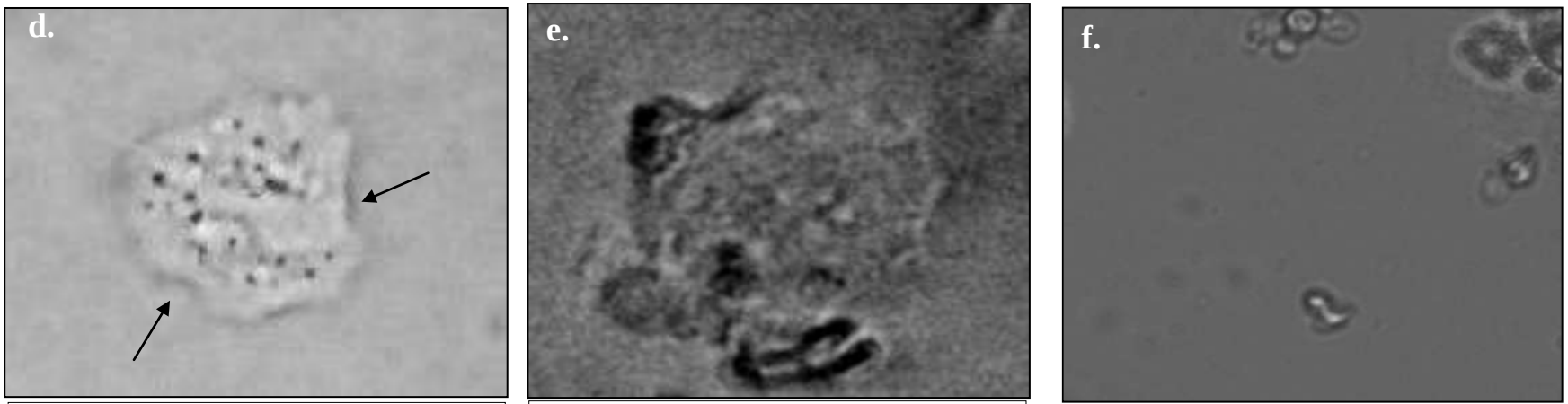
When the growth medium was retained in its standard conditions with inoculum size of 10^4 *T. vaginalis*/ml, amoeboid forms were detected. The size and shapes of the amoeboid forms were slightly smaller and irregular. Maximum length and width of amoeboid forms achieved in a non-stress condition was approximately 17.5 µm and 12.5 µm respectively. Within 24 hours of culture, the trophozoites were observed to move freely and were actively twitching the flagella which had an approximate length of 10 µm. When the incubation period increased to 24 hours, the ovoid trophozoites slowly turned into irregular shape with small part of the cell membrane seen to protrude from one end of the cell. Motility of trophozoite decreased gradually after 24 hours with slow twitching of flagella. The shape became irregular, measuring about 15 µm in length. At 72 hours, the trophozoites transformed into larger, flattened and thin amoeboid forms with inactive flagella. At this point, more trophozoites were observed to be in close contact but in a relaxed state. Slight protrusion of the cytoplasm occurred forming small pseudopodia-like cytoplasmic extensions with few inclusion-like bodies in the cytoplasm.

In contrast, when the growth medium was inoculated with 10^5 *T. vaginalis*/ml, a rapid and significant formation of amoeboid forms was seen. Numbers of trophozoite

forms increased immensely within few hours of culture and were seen to move in groups. Although the trophozoite was seen to move actively, however their movement became restricted with the large number of *T. vaginalis* aggregating into clumps of trophozoites which showed slow motility when observed at six hours of culture. At 12 hours, trophozoites were observed to attach with each other. Few irregular shaped forms were noted to attach with one another and these parasites were motile with slow moving inactive flagella. Amoeboid forms with extensive projection of cytoplasm forming pseudopodia around the membrane were seen. These forms had more inclusion-like bodies and inactive flagella. Fully non-motile amoeboid forms measured approximately 27.5 µm in length and 25 µm in width which was relatively larger than those observed in stress-free medium were only observed in 72 hours culture. The pyriform shaped trophozoites were seen to transform into thin and flattened amoeboid forms. The morphological structure of amoeboid form complete with the pseudopodia-like cytoplasmic extensions and inclusion-like bodies disintegrated after 96 hours and were totally absent in 120 hours culture.



6 hour | 24 hours | 48 hours



72 hours | 96 hours | 120 hours

20 µm

Figure 4.14: a-f Light micrograph showing transformational changes of *T. vaginalis* from trophozoite to amoeboid forms in non-stressed culture conditions. Observation taken from six hours (day 0) up to 120 hours (day 5) in CN isolates. Magnifications 400X.

a Trophozoite of *T. vaginalis*. Note the tear-drop shaped, with active flagella at the anterior end of the cell with length about 8µm at six hour.

b Trophozoite tend to become irregular in shape after 24 hours in the suspension culture. Note that the cell membrane protrudes at one end giving the trophozoite a slightly irregular shape measuring about 10 µm in length.

c Amoeboid forms were seen to form after 36 hours in culture with more irregular in shape. Note that the tear-drop trophozoite has taken a new shape with less active flagella and measuring about 15.5 µm in length.

d Complete formation of viable amoeboid shape was seen at 72 hours. Note the pseudopodia-like cytoplasmic projections (*arrows)*, no flagella, and few inclusion-like bodies (black dots) within the amoeboid cells. The shape was observed to be flattened and larger than the trophozoite and measuring about 17.5 µm in length.

e-f Amoeboid forms with an approximate length of 12 µm start to disintegrate after 96 hours and are totally absent from the suspension culture at 120 hours.

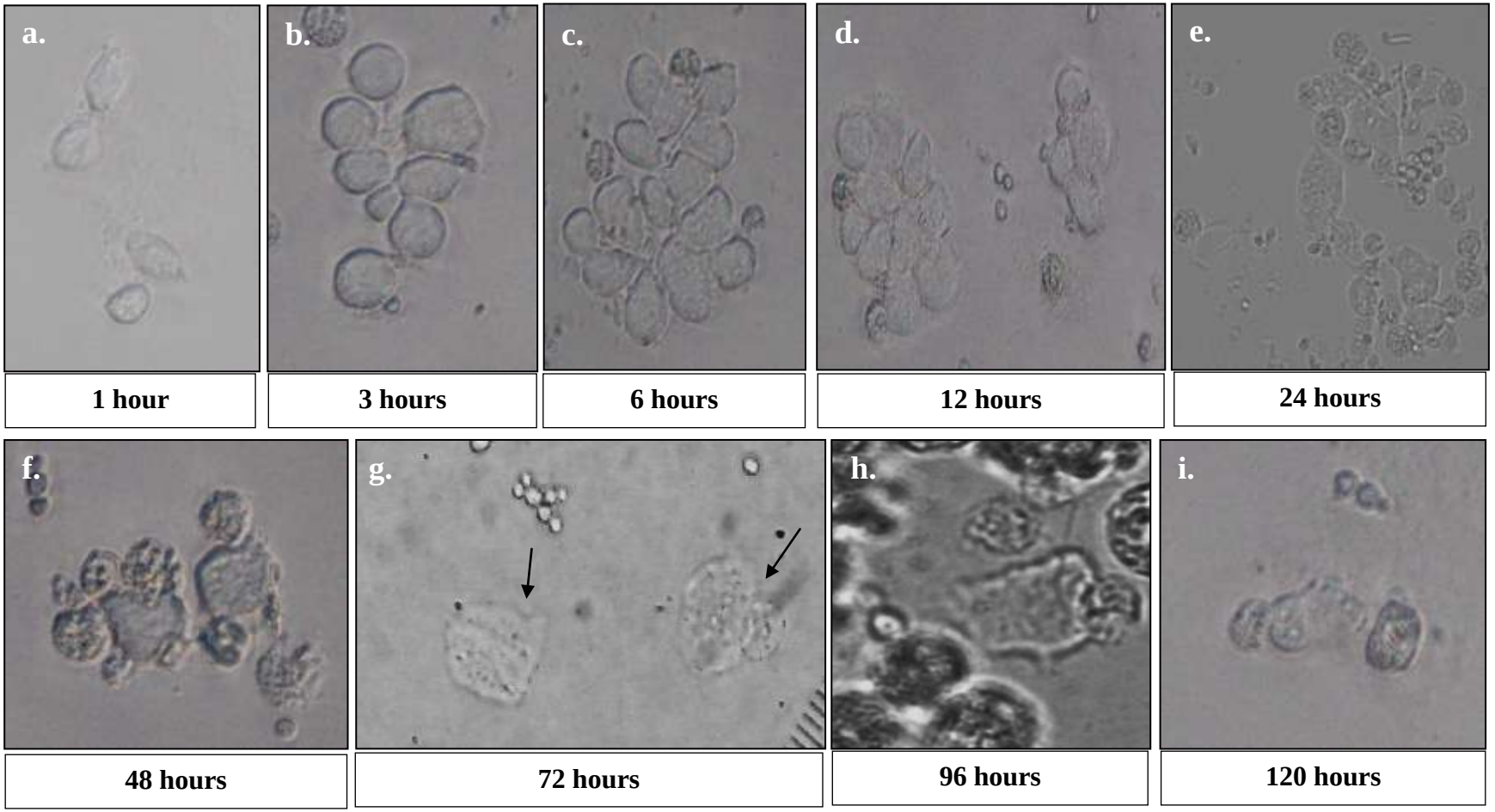

a.
b.
c.
d.
e.
1 hour
3 hours
6 hours
12 hours
24 hours
f.
g.
h.
i.
48 hours
72 hours
96 hours
120 hours
20 µm

Figure 4.15: a-i Light micrograph showing the transformation of *T. vaginalis* from trophozoite to amoeboid form in the suspension culture under the effect of increased cell concentration (10^5 *T. vaginalis*/ml) from one hour (day 0) up to 120 hours (day 5) in CN isolates. Magnifications 400X.

a-d Trophozoites with an average length of 10 µm was observed to come into close proximity due to high number of parasites with increasing culture time (zero hour to six hours) and was completely attached to one another forming big clumps at 12 hours.

e Trophozoites start to change into larger and irregular and seen as partial amoeboid forms with an approximate length of 22.5 µm after 24 hours culture .

f-g Full formation of amoeboid forms with approximate length of 27.5 µm was observed after 48 hours in culture with distinct amoeboid forms showing pseudopodia-like cytoplasmic projections (*arrows*), flattened, appeared larger with more inclusion-like bodies (black dots) at 72 hours culture.

h-i Amoeboid forms was still observed at 96 hours culture but with the cell membrane losing the integrity of the ovoid shape and inclusion-like bodies became less visible. These forms measured an approximate length of 22.5 µm. Amoeboid form was completely absent from the suspension culture after 120 hours culture.

CHAPTER 5

DISCUSSION

5.1 General discussion

In this research, a total of nine symptomatic isolates of *T. vaginalis* consisting of three non-cervical neoplasia (NCN2-NCN4) and six cervical neoplasia (CN1-CN6) were investigated for formation of amoeboid forms under laboratory conditions. The isolates were consistently maintained in axenic Hollander medium to prevent contamination with bacteria or fungi that can interfere with the growth rate of *T. vaginalis*. All the isolates were subjected for growth profiling studies by measuring the number of trophozoite and amoeboid forms. Isolates without amoeboid forms were eliminated for the subsequent component of the study where assessment of the effects on the parasite by changing the culture conditions was carried out. Growth curve is defined as a measurement to study the growth of microorganisms in terms of parasite number (Willey et al., 2009). Therefore, in this study, growth curve reflecting the number of trophozoite and amoeboid forms of *T. vaginalis* was generated to measure the number of these organisms developed with increasing incubation period in order to determine the optimal growth conditions required to produce the maximum number of parasites. All the CN (CN1-CN6) and NCN (NCN2-NCN4) isolates showed a significant growth pattern for the trophozoite forms, however only CN isolates (CN1-CN6) were observed to have the amoeboid forms which will be discussed in the following sub-sections. Variation of growth conditions such as parasites concentration, horse serum concentration and metronidazole concentration were analyzed to determine the effects towards the development of amoeboid forms. Each of these factors is known to have significantly contributed towards the transformational changes of trophozoites to amoeboid forms.

5.2 Observation of amoeboid forms of *T. vaginalis* in suspension culture

The morphology of the amoeboid forms has long been observed as an inherent form of *T. vaginalis* associated with the pathogenic mechanisms (Heath, 1981). Previous studies have shown the appearance of amoeboid forms upon attachment with vaginal cells, blood cells or on cover slips (Arroyo et al., 1993; Brugerolle et al., 1996; Fiori et al., 1999; Tasca & De Carli, 2002). Amoeboid forms were also said to have been triggered upon contact with laminin and fibronectin suggesting a common signaling mechanism through adhesions (Crouch & Alderete, 1999). In axenically grown cultures, Honigberg and Brugerolle have only observed ellipsoid and spheroid forms of *T. vaginalis* (as cited in Jesus et al., 2004). During the phagocytosis process, the virulent strain of *T. vaginalis* changed into the amoeboid shape during internalization of yeast cells (Pereira-Neves & Benchimol, 2007). However, only recently Afzan and Suresh (2012a) proved the formation of this distinctive morphological form of *T. vaginalis* in a suspension culture using transmission and scanning electron microscopy. This study also observed the formation of amoeboid forms in an axenic suspension culture without any types of attachments.

Morphologically, the amoeboid forms were observed as flattened, non-motile or slowly motile with inactive flagella and a delicate outer layer with projecting pseudopodia-like cytoplasmic extensions when attached to epithelial or vaginal cells (Heath 1981; Fiori et al., 1999; Tasca & De Carli, 2002). Abnormal forms of *T. vaginalis* has also been identified with larger size compared to normal cell size with spiky projections on irregular surfaces and multiple stumps and a fragmented undulating membrane (John & Squires, 1978b). Rasmussen et al. (1986) observed an amoeboid form with a pseudopodium projected into the cytoplasm of disintegrated epithelial cells. The present study concurred with previous findings which showed that the amoeboid forms had multiple projections of the pseudopodia, non-motile or slow motile, none or

inactive flagella, higher numbers of inclusion–like bodies, flattened, bigger and irregular in shape. However, these forms only occurred under stressed conditions in suspension cultures as identified by Afzan and Suresh (2012a). Nevertheless, there has been no report on the numbers of amoeboid forms in cultures and factors elucidated to trigger these forms to exist in suspension cultures. Thus, this study is the first to identify factors that contribute to the formation of amoeboid forms under certain culture conditions.

5.3 Growth profiles of amoeboid forms of *T. vaginalis*

The maximum count of amoeboid forms was seen on day 3 in suspension cultures of CN isolates. No amoeboid forms were seen in NCN isolates of *T. vaginalis.* The results concurred with findings of Afzan and Suresh (2012a). However the present study observed amoeboid forms on day 1 for CN3 and CN5, day 2 and day 4 for all CN isolates with a lower count of $0.66 \pm 0.17 \times 10^4$ *T. vaginalis*/ml, $5.67 \pm 0.25 \times 10^4$ *T. vaginalis*/ml and $2.99 \pm 0.28 \times 10^4$ *T. vaginalis*/ml respectively. Although the amount of amoeboid forms detected in the culture was relatively low, it was indeed higher compared to the findings by Afzan and Suresh (2012a). This could be due to the prolonged cultivation of the CN isolates that enabled them to adapt to the growth medium.

Amoeboid forms were seen to appear along with the trophozoites of *T. vaginalis* and were at optimum when the numbers of trophozoites peaked on day 3 in all CN isolates. The formation of amoeboid forms corresponds to the number of trophozoites of *T. vaginalis*. The higher the number of trophozoites, greater is the probability of seeing amoeboid forms in the culture. This is because as more numbers of trophozoites are produced in the culture medium, the limited space and the overcrowding facilitates contact with one another which could be a factor to trigger the formation of amoeboid forms.

Amoeboid forms have been persistently observed in subsequent sub-cultures following first or second passage but in inconsistent numbers and always intermixed with pyriform trophozoite (Tasca & De Carli, 2002). Afzan and Suresh (2012a) reported that amoeboid forms were detected only on day 3 when the parasites were overcrowded and in frequent contact with each other. The present study also observed optimum numbers of amoeboid forms on day 3 when the trophozoites were high in number. However, the amoeboid forms were still detected on day 1, day 2 and day 4 of certain CN isolates suggesting that other factors such as cultivation period and adherence effect to glass surface as well as unfavourable culture conditions could have triggered the formation of the amoeboid forms in these isolates.

Amoeboid forms were not observed after 96 hours (day 4) of culture but viable and motile trophozoites were seen instead but in lower numbers. Present findings indicate that the amoeboid forms are an integral part of the life cycle of *T. vaginalis* and they appear to emerge in crowded cultures where the parasites are forced to be in close contact with one another. In another study the virulence factor was said to be significant in triggering amoeboid forms more in prolonged isolates than fresh isolates (Tasca and De Carli et al., 2002).

Some studies have shown that well-established *T. vaginalis* strains when maintained for years under laboratory conditions were less adherent (Rasmussen et al., 1986), had low virulence and without amoeboid morphology (Jesus et al., 2004). Tasca and De Carli (2002) have reported that more virulent strains tend to settle on and adhere to the culture cells compared to less virulent strains due to prolonged cultivation. In spite of these observations, the present study has shown that the amoeboid forms of *T. vaginalis* from CN isolates that has been cultured for a long time can be found *in vitro* and without induced attachments to vaginal epithelial cells or human erythrocytes. Jesus et al. (2004) have also observed amoeboid forms in fresh culture medium without

incubation of host cells but in freshly isolated strains of *T. vaginalis*. Therefore, the present finding has opened a new prospect of finding amoeboid forms *in vitro* in medium free of host cells and in prolonged cultivated state. Amoeboid forms in trichomonasis patients with cervical cancer could play a role in exacerbating the cervical cancer condition.

5.4 Growth profile of trophozoite forms of *T. vaginalis*

Study on growth patterns of the trophozoite forms of *T. vaginalis* have been performed by many investigators (Lehker & Alderete, 1990; Kostara et al., 1998; El-Okbi et al., 2004; Boulos et al., 2012). Most of the growth profile studies conducted were as a part of phenotypic characterization studies to evaluate the viability of the trophozoites in *in vitro* growth conditions (Cox & Nicol, 1973; Lehker & Alderete, 1990), to elucidate the growth rate of trophozoites in different growth media (Gelbart et al., 1990; Ryu et al., 1998) as well as to understand the metabolic activities based on the growth profile of *T. vaginalis* from CN isolates and NCN isolates (Yusof and Kumar 2012). However, the present study on growth profile of trophozoites was performed from the same samples obtained from the study carried out by Yusof and Kumar (2012) with an inoculum size of 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml. Inoculum size of 10^4 *T. vaginalis* /ml was used as the base line to compare growth rates with other inoculum sizes.

The present study concurred with the finding of Yusof and Kumar (2012) that significant optimal growth rate of parasites in all CN isolates on day 3 of culture were seen with trophozoite number to be $224.22 \pm 2.19 \times 10^4$ *T. vaginalis*/ml. However, the results differed with NCN isolates as NCN2 and NCN3 showed peak growth on day 4 while NCN4 was on day 3 with mean trophozoites count of $88 \pm 1.41 \times 10^4$ *T. vaginalis*/ml and $92.33 \pm 4.93 \times 10^4$ *T. vaginalis*/ml respectively. The peak growth on day 3 in the present study was comparable to a study carried out on five clonal strains

of *T. vaginalis* which observed a similar pattern of growth profile for trophozoites with highest count on 72 hours of culture (Chaudhari & Singh, 2011).

The average trophozoites count obtained for both CN and NCN isolates in this study was relatively higher than that shown in the study of Yusof and Kumar (2012). This could be due to the good adaptation of the isolates to the growth conditions using Hollander medium. There were great variation between the average count of trophozoites in CN and NCN isolates with the former having higher trophozoites count. This could be due to the difference in biological and metabolic activities. Trophozoites from CN isolates could be more vibrant and active than NCN isolates because of a higher virulence nature of the cells. The growth rate of trophozoites in CN and NCN isolates were dependent on the growth conditions such as nutrient composition, temperature and pH (Kostara et al., 1998).

Combined growth profile of trophozoites for CN isolates with initial inoculum of 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml observed no significant mean difference of trophozoites detected in cultures with 10^4 and 10^5 *T. vaginalis*/ml. However the growth rate was observed to be higher in culture medium with higher inoculum size of parasite. Both types of inoculum sizes reported a peak growth for all CN isolates on day 3 with mean count of 224.22 ± 2.19 x 10^4 *T. vaginalis*/ml (37.22 %) and 429.22 ± 11.19 x 10^5 *T. vaginalis*/ml (45.99 %) respectively. The numbers of trophozoites detected in cultures with inoculum size of 10^5 *T. vaginalis*/ml were higher in cultures from day 1 to day 4 than cultures with inoculum size of 10^4 *T. vaginalis*/ml probably observed during the same period. This is due to the higher propagation rate seen in cultures with larger inoculum size. Similarly, Kulda et al. (1970) using higher initial inoculum of 7 x 10^5 *T. vaginalis/*ml reported that fast growing strains of *T. vaginalis* reached maximum growth after 32 hours compared to 48 to 52 hours for slow growing strains (Boulos et al., 2012).

Growth profile study of trophozoite forms has been show to imply that essential growth conditions are essential for the prolonged survival of *T. vaginalis* (Kostara et al., 1998). Besides, the growth profile of trophozoites was comparable with the growth profile of amoeboid forms in CN isolates. This is because, for both type of inoculum sizes, when the trophozoites count were optimum on day 3, the amoeboid forms are also optimum.

5.5 Assessment of staining methods for the detection of amoeboid forms

Modified Fields' staining have been shown to be a good rapid assay for the detection of *T. vaginalis* and was preferred over Giemsa and Gram stain (Afzan et al., 2010) for this study . The staining of trophozoites of *T. vaginalis* following the method of Afzan et al. (2010) showed that the nucleus and cytoplasm stained reddish purple and light purple respectively and was detected in 20 seconds. The amoeboid forms detected in the present study using Modified Field's stain stained the nucleus and cytoplasm with brighter and lighter reddish purple and was effectively seen when stained within 15 seconds. The small differences in the colour of the organelles and time could be due to the large size of amoeboid forms which caused them to be stained with intense colouration. The easy and reliable preparation of Fields' stain was preferred over time-consuming Giemsa stain. Besides being useful for the detection of amoeboid forms of *T. vaginalis*, Modified Fields' stain elucidated greater morphological details especially the biology of the multiple nuclei formation seen in some of the amoeboid forms (unpublished observation).

5.6 Effect of different stress conditions on the development of amoeboid forms

Based on the study conducted to analyze the effects of varying the culture conditions in triggering the formation of amoeboid forms of *T. vaginalis*, the present results have verified the assumptions made by Afzan and Suresh (2012a). This is on the possible role of the stress applied on the culture conditions to trigger the transformation of the motile trophozoites to non-motile amoeboid forms. All the three culture conditions studied were able to induce amoeboid forms in the CN isolates of *T. vaginalis* to a certain extent. *T. vaginalis* is an opportunistic organism that takes advantage of the changing surrounding conditions in the human genitourinary tract for continual survival. The efficient growth potential of *T. vaginalis* in an *in vitro* condition is primarily due to the availability of sufficient nutrients and suitable growth environment in the suspension culture that allows the parasite to proliferate and expand its infection (Figueroa-Angulo et al., 2012).

A deviation from the standard growth conditions in terms of varying the nutrient supply, increasing the parasites count in the medium and introducing growth suppressing drug affected the adaptability and survival rate of *T. vaginalis*, thus signaling the development of the amoeboid forms. Present study analysed three different growth conditions based on number of parasites, horse serum concentrations and effect of metronidazole drug which showed significant formation of amoeboid forms in six CN isolates for the respective conditions. Previous studies have analyzed the effects of growth environment on the virulence and survival rate of *T. vaginalis* but only on the trophozoite forms (Lehker & Alderete, 1990; Kostara et al., 1998; Figueroa-Angulo et al., 2012). Present study was the first to demonstrate the effects of growth conditions manipulation in triggering amoeboid forms of *T. vaginalis*.

5.6.1 Effect of different inoculum sizes

Increasing the inoculum size from 10^4 *T. vaginalis*/ml to 10^5 *T. vaginalis*/ml caused a significant difference in triggering amoeboid form ($p < 0.05$). Concurrently, the amount of trophozoites was significantly higher in inoculums size of 10^5*T. vaginalis*/ml than in 10^4 *T. vaginalis*/ml. Amoeboid forms were not seen in culture of CN isolates with parasite concentrations of 10^3 *T. vaginalis*/ml and 10^6 *T. vaginalis*/ml. This could mean that too low or too high inoculum sizes fail to facilitate the triggering of these forms. A very low amount of inoculum size reduces the opportunity for cell contact between the cells whereas the higher inoculum size may use up the nutrients faster and thereby prevent the triggering of *T. vaginalis* to form amoeboid forms. Only inoculum between 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml showed a significant number of amoeboid forms. Hence it appears that there is a balance requirement between the numbers of trophozoites in the media and availability of sufficient nutrients to create frequent collision between each parasite to form the amoeboid forms.

Boulos et al. (2012) showed that in their study the fast growing isolates had higher rate of division. When the growth conditions were stressed with overcrowding of parasites in one ml, the motility of trophozoites were inhibited due to limited space for growth resulting in more frequent contact between the parasites leading to the appearance of more number of amoeboid forms as seen in cultures with inoculum sizes of 10^5 *T. vaginalis*/ml when compared to 10^4 *T. vaginalis*/ml. As the number of trophozoites reduced with increasing incubation period, the amoeboid forms were also reduced implying that overcrowding is a factor in triggering this abnormal shape of *T. vaginalis*.

The situation can be extrapolated to a human urogenital system infected with trichomonas infection. In the initial stages, the parasites multiply and colonize a wide area and in cases with patients having CN, the parasites might reproduce rapidly due to

changes in the environment and potentially give rise to pathogenic amoeboid forms. Eventually, the infection could be exacerbated with these forms contributing to the complication in infected women.

5.6.2 Effects of different horse serum concentrations

Nutritional compositions in the growth medium of CN isolates of *T. vaginalis* also played an important role in triggering amoeboid forms as seen from the results obtained from the effects of different horse serum concentrations. Inside the host, *T. vaginalis* acquires the essential growth factors from vaginal secretions or through phagocytosis of the vaginal microflora (Petrin et al., 1998). Likewise, *T. vaginalis* must be supplied with the necessary nutrients in order to maintain the survivability. Thus this experiment assessed if nutrient depletion in the *in vitro* condition can trigger amoeboid forms. In this study, the Hollander medium used for growth of *T. vaginalis* was supplemented with heat-inactivated horse serum as the main source of nutrients. Horse serum is a donor-herd collected serum that contains higher protein concentration with essential growth factors, vitamins, co-factors, hormones, attachment factors, transport factors, nutrients and trace elements to support an effective growth rate in *T. vaginalis* ("*Sera for cell culture*," n.d). A number of studies have described the possible effects of nutrients such as iron (Kim et al., 2006) and polyamines (Figueroa-Angulo et al., 2012) in regulating the growth metabolism of *T. vaginalis*.

Varying the concentration of horse serum in the growth media of *T. vaginalis* was observed to significantly affect the growth potential of trophozoite forms in CN isolates which indirectly influenced the development of amoeboid forms. Lowest concentration of horse serum of 1 % reported zero formation of amoeboid forms due to limited-availability of nutrients to support the growth of *T. vaginalis*. The mobility and viability of trophozoites in this medium was extremely poor and deteriorated within 24 hours of incubation. Since amoeboid forms were associated with trophozoites, therefore

lower nutrients contents affected their potential development. The findings on the role of nutrient deficiency in controlling the cell viability concurred with the study by Lehker and Alderete (1992) on the fact that the depletion of a nutrient source primarily iron can affect the growth rate of *T. vaginalis.* They concluded that iron limitations resulted in decrease in protein synthesis and cell densities of *T. vaginalis*. Virulence of *T. vaginalis* was also reported to be reduced with reduction of iron in the growth medium (Ryu et al., 2001).

As the horse serum concentration was increased to 5 %, calculated mean average amoeboid forms was 3.95 ± 0.18 x 10^4 *T. vaginalis*/ml and increased slightly to 4.33 ± 0.18 x 10^4 *T. vaginalis*/ml with 10 % of horse serum. Optimal amoeboid count was obtained when the horse serum was 15 % and decreased gradually when the horse serum concentration was increased to 20 %, reflecting the role of adequate protein content in triggering this shape. Higher hyrogenosomal activity of *T. vaginalis* have been reported in iron enriched medium compared to iron depleted medium, thus indicating the importance of having sufficient nutrients for optimal growth of parasites (Kim et al., 2006). Horse serum concentration of 15 % was the impulse for active formation of amoeboid forms of *T. vaginalis* by providing sufficient amount of nutrients for the growth of trophozoites which then proliferated rapidly and vigorously until they gained opportunities to transform into amoeboid forms.

5.6.3 Effects of different concentrations of metronidazole drug

In the final evaluation of stress conditions on amoeboid formation, effects of metronidazole drug were tested against CN isolates to determine if metronidazole can trigger the amoeboid forms. Metronidazole was chosen as the drug of choice due to its effectiveness in treating *T. vaginalis* infection although few cases of metronidazole resistance have been reported (Meri et al., 2000; Chaudhari & Singh, 2011). The findings revealed that the first three highest concentration of metronidazole drug used; 1

mg/ml, 0.1 mg/ml and 0.01 mg/ml reported no formation of amoeboid forms because all the trophozoites were highly susceptible to these concentrations and died immediately within hours after cultures were treated. This clearly explains the close association of amoeboid forms with trophozoites whereby the dominant pyriform must be present in order for the irregular amoeboid form to develop.

When the concentration was slightly lowered to 0.001 mg/ml and 0.0001 mg/ml, amoeboid forms were observed to appear in the suspension culture with the highest count seen in cultures treated with 0.0001 mg/ml. This suggests that the CN isolates of *T. vaginalis* at lower doses of the drug triggered steady growth of trophozoites which then lead to the formation of amoeboid forms. The higher resistance of CN isolates to metronidazole drug up to 100 µg/ml in a previous study reflected the parasite from CN isolates were robust (Afzan, 2011d). This caused the parasites to divide rapidly and transformed into non-motile amoeboid forms again pointing to the idea that close contact with cells could be a factor in inducing the parasite to form amoeboid forms.

In cervical cancer patients the parasites can attach to vaginal cells or epithelial cells and transform into amoeboid forms which can increase the severity of the infections with *T. vaginalis*. Formation of amoeboid forms upon attachment to cover slips have been demonstrated with actin cytoskeleton activity contributing to the adhesion and phagocytic ability (Brugerolle et al., 1996).

Lower drug concentration of 0.001 mg/ml and 0.0001 mg/ml triggered a proliferation of the parasite compared to the untreated culture (control) and produced significant high parasite count ($p < 0.05$). Higher concentration of metronidazole however killed *T. vaginalis*. This suggests that if patient stops their medication half way during treatment or if the drug dose is insufficient this can be more harmful as it can trigger higher parasite number. High dosage of 2.6 g of metronidazole/day has been given for a period of nine days to patients with refractory vaginal trichomoniasis but

reported a number of failure cases due to resistance effect (Lossick et al., 1986). Conversely, a regimen of 400 mg metronidazole twice daily for seven days were found to be effective when taken in prescribed dosage (Keighley, 1971). The present study is crucial as it contributes to the idea that correct treatment dose is of paramount importance in treating *Trichomonas*-infected patients with cervical cancer.

5.7 Significance on the effects of stress factors in triggering amoeboid forms

In all the three culture conditions studied, it was anticipated that stress factors in the growth culture medium of *T. vaginalis* will most likely trigger amoeboid forms when culture conditions become unfavourable. Although stress-free cultures still report the presence of amoeboid forms, however the numbers were far less when compared with stressed cultures. Overall, amoeboid forms are triggered when the trophozoites grow in high numbers causing a lack of nutrients as well as enhancing contact with one another due to overcrowding. However, it must be noted that in every stressed condition tested, as long as the numbers of trophozoite significantly increase there is likelihood that amoeboid forms can be triggered. Factors such as different concentrations of horse serum and metronidazole merely facilitated the propagation of parasites in culture. Higher numbers of trophozoite increased cell contact and this triggered higher number of amoeboid forms seen in cultures.

Previous studies have shown that the amoeboid forms seen when the cytoadherence mechanisms aided by adhesin proteins got triggered when attaching to surfaces (Arroyo et al., 1993; Fiori et al., 1999; Moreno-Brito et al., 2005; Ryan et al., 2011). There have been previous studies to suggest that a virulence property in T. vaginalis in triggering amoeboid forms was achieved when culture conditions were changed (Arroyo et al., 1993; Fiori et al., 1999; Liang & Huang, 2009).

5.8 Transformational changes analysis

In the description of phenotypic characteristics of amoeboid forms, the transformation of the trophozoites into the amoeboid forms were measured based on size and shape. Transformational changes of pseudocysts forms were shown revert to trophozoites when lysed with distilled water (Afzan and Suresh, 2012b). However, this is the first study to show the morphological changes from actively swimming trophozoites into non-motile amoeboid forms. Based on the present findings, it can be further confirmed that the amoeboid forms of *T. vaginalis* do appear in suspension cultures unlike previous reports where these forms were only seen when grown on cover slips containing epithelial or erythrocytes cells.

The significant difference seen between the amoeboid formation in a stress-free and stressed culture medium described the role of culture conditions in influencing the formation of these life cycle stages.

Trophozoites in stressed-culture lose their motility earlier (within 48 hours) compared to trophozoites in control tubes (after 48 hours) due to the probable dense parasite numbers in cultures which restrict their movements. The irregular shape of amoeboid structures could be formed to gain attachment. The pseudopodia-like cytoplasmic extensions were also observed to appear on day 3. Similar to the present observation, Arroyo et al. (1993) captured a similar transformation of globular shape parasites into thin, flat amoeboid forms when in contact with vaginal epithelial cells. Ryan et al. (2011) also visualized the transition of ovoid free-swimming forms of *T. vaginalis* into amoeboid forms upon binding to a host epithelial cell.

Conversion of trophozoites to amoeboid forms saw the development of more inclusion-like bodies suggesting that there is an active process involved during the transformation. These inclusion-like bodies could be glycogen granules found in the cytoplasm of *T. vaginalis* since evidence have shown rich contents in these vesicles in

the parasite as observed through electron microscopy (Costamagna & Figueroa, 2001). The name itself reflects the role of these granules as food storage. It is possible that inclusion-like bodies in amoeboid forms may play role in synthesizing energy to trigger activities.

The present study confirmed that stressful conditions can trigger amoeboid forms in culture. More molecular studies need to be carried out to better understand the role of the amoeboid forms.

CHAPTER 6

CONCLUSION

Isolates of *T. vaginalis* diagnosed from patients with cervical neoplasia are potential for the development of amoeboid forms as they are more likely to be triggered under various growth conditions. High number of trophozoites is the critical factor for the formation of amoeboid forms. At normal culture condition, highest numbers of amoeboid forms was seen in day 3 culture. The study for the first time detected amoeboid forms in suspension culture using Modified Fields' stain with significant extensions of cytoplasm to form the pseudopods, rough surfaces and with single or multiple nuclei. The study also for the first time demonstrated the phenotypic characteristics of amoeboid forms in suspension cultures obtained from stressed and non-stressed conditions.

A potential application of this study would be to identify the dietary intake of patients diagnosed with cervical neoplasia and trichomoniasis, and ascertain the severity of symptoms with the type of food intake. An in depth understanding of the disease may help to lower the clinical symptoms of the infection and prevent further complications. Other potential growth parameters such as temperature and pH can also be varied to identify their effects in triggering amoeboid forms. A prospective study by looking at the gene and protein expression of the amoeboid forms can also be initiated to determine if these forms can be identified in suspension cultures. In addition, a better observation on the transformational changes taking place from trophozoite to amoeboid forms must be performed to better understand the process taking place.

Based on the findings of this study, the following conclusions were drawn:

1. The cultures used in the present study were four years old indicating that the isolates can be maintained for a long time without the parasite losing its ability to trigger amoeboid forms.

2. Amoeboid forms were only detected in CN isolates with higher count on day 3 with average amoeboid of 28.66 ± 7.93 x 10^4 *T. vaginalis*/ml. The amoeboid forms were observed with pseudopodia-like cytoplasmic extension, none or inactive flagella, non-motile, irregular in shape, were larger than trophozoites, flattened and with more inclusion-like bodies.
3. Cultures of CN showed higher growth rate of trophozoites than those of NCN isolates in inoculum concentration of 10^4 *T. vaginalis*/ml with total average count of 3615.04 ± 67.69 x 10^4 *T. vaginalis*/ml.
4. Total average number of trophozoites was higher in culture with inoculum size of 10^5 *T. vaginalis*/ml compared to inoculum size of 10^4 *T. vaginalis*/ml. The numbers of trophozoite in culture with 10^5 *T. vaginalis*/ml for all CN isolates were 933.28 ± 18.71 x 10^5 *T. vaginalis*/ml.
5. Modified Fields' stain can be efficiently used to detect amoeboid forms of *T. vaginalis* with clear morphological details such as multiple or single nuclei as well other cellular details.
6. 15 % horse serum and an inoculum size of 10^5 *T. vaginalis*/ml yielded the highest amoeboid forms with average count of 59.67 ± 2.80 x 10^4 *T. vaginalis*/ml and 116.65 ± 3.96 x 10^5 *T. vaginalis*/ml respectively showing that the interaction between the parasites and the correct nutrient amount appears to be key factors for triggering amoeboid forms.
7. Lowest drug concentration of 0.0001 mg/ml (21.66 ± 0.39 x 10^4 *T. vaginalis*/ml) triggered these forms implying that metronidazole used for the treatment of trichomoniasis when not taken at the right dose can trigger more amoeboid forms to develop.
8. The study confirmed that amoeboid forms were triggered due to stress response when culture conditions were altered. There is a possibility that chemotherapy

drugs and different food intake during treatment of cervical cancer patients infected with the parasite can trigger these forms. The exact role of these forms has yet to be elucidated but the possibility of cell invasion cannot be ruled out which in turn can exacerbate the already complicated condition. The summary of comparison of amoeboid forms in stress and non-stress conditions is presented in Table 4.12.

9. Clumping of trophozoites was observed as the first step leading to amoeboid formation in suspension culture under stress conditions. Parasite clumping is due to the high number of trophozoites. This facilitated contact with one another which eventually triggers to form as amoeboid forms.

Overall, the present findings were able to answer the research questions asked at the beginning of the study. There are as follows:

i. The present study concurs with the findings of Afzan and Suresh (2012a) that amoeboid forms do exist and they are found in CN isolates predominantly on day 3 cultures. Despite 4 years of age the cultures were consistent in producing the amoeboid forms.

ii. Stressing the conditions of the growth medium does trigger the formation of amoeboid forms in suspension culture.

iii. The morphology that differentiates amoeboid forms are the pseudopodia-like cytoplasmic extension, none or inactive flagella, non-motile, irregular in shape, larger than trophozoites, flattened and more inclusion-like bodies.

Table 4.12: Summary of comparison of amoeboid forms between stressed conditions and normal conditions.

Features	Amoeboid forms	
	Stressed conditions	**Normal conditions (stress-free)**
Size	Larger, 27.5 µm (length) and 25 µm (width)	Smaller, 17.5 µm (length) and 12.5 (width)
Shape	Irregular with more pseudopodia-like cytoplasmic extensions	Irregular with less pseudopodia-like cytoplasmic extensions
Number of inclusion bodies	More (25 to 30 granules in one cell)	Less (15 to 17 granules in one cell)
Number of amoeboid forms	Higher, 116.65 ± 3.96 x 10^5 *T. vaginalis*/ml	Lower, 28.66 ± 7.93 x 10^4 *T. vaginalis*/ml
Arrangements of trophozoites	Appeared in clumps	Loosely or individually distributed

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Appendix A

Clinical identity of each isolates from CN and NCN cultures as obtained by Afzan (2011c).

Table 1: Clinical identity of the samples

T.vaginalis positive	**Race**	**Age (years old)**	**Marital status**	**Multiple/single partner**	**Inflammation**	**Cervical neoplasia (CIN3)**	**Reported symptoms of patients**
NCN1	Malay	32	Single	Multiple	Moderate	No	Heavy discharge
NCN2	Malay	30	Married	Single	Moderate	No	Heavy discharge
NCN3	Chinese	48	Single	Multiple	Moderate	No	Heavy discharge
NCN4	Malay	25	Married	Single	Moderate	No	Heavy discharge
CN1	Chinese	49	Married	Single	Moderate	Yes	Heavy discharge, punctate strawberry spots
CN2	Chinese	52	Married	Single	Moderate	Yes	Abdominal distension, punctate strawberry spots
CN3	Chinese	52	Married	Single	Moderate	Yes	Creamy discharge, abdominal distension, punctate strawberry spots
CN4	Chinese	45	Married	Single	Moderate	Yes	Frothy discharge, punctate strawberry spots
CN5	Chinese	31	Single	Multiple	Moderate	Yes	Greenish yellowish discharge, abdominal distension, punctate strawberry spots
CN6	Malay	52	Married	Single	Moderate	Yes	Heavy discharge, itching abdominal distension, punctate strawberry spots

Appendix B

Hollander medium preparation for cultivation of *T. vaginalis*

Tryptic Soy Broth (TSB)	20.0 g
Yeast extract	10.0 g
Maltose	5.0 g
Ascorbic acid	1.0 g
KCl	1.0 g
KH_2PO_4	1.0 g

All the ingredients mentioned above are dissolved in 900 ml of distilled water and autoclave for 15 minutes at 121 ºC. Once cooled, the medium is stored at 4ºC. Prior use, 100 ml of heat inactivated horse serum and 20 ml of Penicillin-Streptomycin Neomycin is added to the medium and mixed well.

Appendix C

Statistical analysis results to test the significance of amoeboid forms in CN isolates of *T.vaginalis* between days.

Test of Homogeneity of Variances

Average amoeboid count x 10^4 *T. vaginalis*/ml

Levene Statistic	df1	df2	Sig.
7.448	4	25	.000

Test Statistics[a,b]

	Average amoeboid count x 10^4 *T. vaginalis*/ml
Chi-Square	26.479
Df	4
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: Days of count

Output:

a. Test of homogeneity of variances : equality of variances not assumed
b. Kruskal-Wallis: $p < 0.05$ – at least one pair of days of average trophozoites forms differs.

Hence, Post-hoc test using Dunnett T3 was performed to identify the days that differ significantly (Table 4).

Table 4: Average amoeboid forms in CN isolates of *T. vaginalis* at 10^4 *T. vaginalis*/ml

Multiple Comparisons

Dependent Variable: Average amoeboid count x 10^4 *T. vaginalis*/ml

Dunnett T3

(I) Days of count	(J) Days of count	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Day 1	Day 2	-.83500*	.12298	.001	-1.2735	-.3965
	Day 3	-3.11333*	.27577	.000	-4.2417	-1.9849
	Day 4	-.44500	.13185	.071	-.9220	.0320
	Day 5	.11000	.06957	.702	-.1900	.4100
Day 2	Day 1	.83500*	.12298	.001	.3965	1.2735
	Day 3	-2.27833*	.28548	.001	-3.3958	-1.1609
	Day 4	.39000	.15110	.200	-.1350	.9150
	Day 5	.94500*	.10141	.002	.5077	1.3823
Day 3	Day 1	3.11333*	.27577	.000	1.9849	4.2417
	Day 2	2.27833*	.28548	.001	1.1609	3.3958
	Day 4	2.66833*	.28941	.000	1.5522	3.7844
	Day 5	3.22333*	.26685	.000	2.0728	4.3739
Day 4	Day 1	.44500	.13185	.071	-.0320	.9220
	Day 2	-.39000	.15110	.200	-.9150	.1350
	Day 3	-2.66833*	.28941	.000	-3.7844	-1.5522
	Day 5	.55500*	.11200	.029	.0721	1.0379
Day 5	Day 1	-.11000	.06957	.702	-.4100	.1900
	Day 2	-.94500*	.10141	.002	-1.3823	-.5077
	Day 3	-3.22333*	.26685	.000	-4.3739	-2.0728
	Day 4	-.55500*	.11200	.029	-1.0379	-.0721

*. The mean difference is significant at the 0.05 level.

Output:

Based on the multiple comparison made, it can be concluded that average trophozoite count on day 3 is significantly higher compared average trophozoites count on day 1, day 2, day 4 and day 5.

Appendix D

Raw data of trophozoite counts in NCN and CN isolates (10^4 *T. vaginalis*/ml).

Isolates : Non-cervical neoplasia (NCN) 2						
Days	**No of trophozoites x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	7	5	4	5.33	1.53	-1.53
2	9	13	11	11.00	2.00	-2.00
3	45	40	39	41.33	3.21	-3.21
4	91	79	97	89.00	9.17	-9.17
5	100	80	79	86.33	11.85	-11.85
6	57	65	68	63.33	5.69	-5.69
7	41	49	46	45.33	4.04	-4.04
8	26	17	15	19.33	5.86	-5.86
9	14	8	21	14.33	6.51	-6.51
10	8	6	10	8.00	2.00	-2.00
11	0	0	0	0.00	0.00	0.00

Isolates : Non-cervical neoplasia (NCN) 3						
Days	**No of trophozoites x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	7	6	5	6.00	1.00	-1.00
2	12	6	12	10.00	3.46	-3.46
3	48	60	43	50.33	8.74	-8.74
4	97	86	78	87.00	9.54	-9.54
5	60	52	50	54.00	5.29	-5.29
6	35	48	71	51.33	18.23	-18.23
7	25	25	31	27.00	3.46	-3.46
8	11	13	8	10.67	2.52	-2.52
9	6	7	3	5.33	2.08	-2.08
10	1	5	3	3.00	2.00	-2.00
11	0	0	0	0.00	0.00	0.00

Isolates : Non-cervical neoplasia (NCN) 4						
Days	**No of trophozoites x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	12	15	12	13.00	1.73	-1.73
2	22	25	37	28.00	7.94	-7.94
3	98	90	89	92.33	4.93	-4.93
4	78	75	70	74.33	4.04	-4.04
5	67	56	68	63.67	6.66	-6.66
6	55	48	35	46.00	10.15	-10.15
7	32	35	30	32.33	2.52	-2.52
8	13	11	10	11.33	1.53	-1.53
9	9	8	7	8.00	1.00	-1.00
10	5	3	3	3.67	1.15	-1.15
11	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 1						
Days	**No of trophozoites x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	16	20	14	16.67	3.06	-3.06
2	99	100	110	103.00	6.08	-6.08
3	211	220	234	221.67	11.59	-11.59
4	89	90	85	88.00	2.65	-2.65
5	70	60	62	64.00	5.29	-5.29
6	52	53	59	54.67	3.79	-3.79
7	25	25	26	25.33	0.58	-0.58
8	15	12	19	15.33	3.51	-3.51
9	5	0	0	1.67	2.89	-2.89
10	1	0	0	0.33	0.58	-0.58
11	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2 (Repeated on 3/11/2012)						
	No of trophozoites x 10^4 *T. vaginalis*/ml				Standard Deviation (±)	
Days	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average counts**		
1	11	17	20	16.00	4.58	-4.58
2	106	101	90	99.00	8.19	-8.19
3	209	189	232	210.00	21.52	-21.52
4	100	85	98	94.33	8.14	-8.14
5	87	73	65	75.00	11.14	-11.14
6	2	20	29	24.67	4.51	-4.51
7	19	14	13	15.33	3.21	-3.21
8	10	9	7	8.67	1.53	-1.53
9	5	4	0	3.00	2.65	-2.65
10	0	0	0	0.00	0.00	0.00
11	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 3						
No of trophozoites x 10^4 *T. vaginalis*/ml					Standard Deviation (±)	
Days	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average counts**		
1	41	52	38	43.67	7.37	-7.37
2	78	89	90	85.67	6.66	-6.66
3	201	221	207	209.67	10.26	-10.26
4	130	168	151	149.67	19.04	-19.04
5	80	79	74	77.67	3.21	-3.21
6	30	29	20	26.33	5.51	-5.51
7	26	20	15	20.33	5.51	-5.51
8	19	12	10	13.67	4.73	-4.73
9	11	6	5	7.33	3.21	-3.21
10	6	0	1	2.33	3.21	-3.21
11	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
No of trophozoites x 10^4 *T. vaginalis*/ml					Standard Deviation (±)	
Days	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average counts**		
1	15	21	15	17.00	3.46	-3.46
2	145	130	159	144.67	14.50	-14.50
3	256	233	231	240.00	13.89	-13.89
4	154	167	190	170.33	18.23	-18.23
5	78	67	61	68.67	8.62	-8.62
6	46	37	39	40.67	4.73	-4.73
7	30	25	20	25.00	5.00	-5.00
8	17	11	9	12.33	4.16	-4.16
9	3	8	2	4.33	3.21	-3.21
10	0	5	0	1.67	2.89	-2.89
11	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
No of trophozoites x 10^4 *T. vaginalis*/ml					Standard Deviation (±)	
Days	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average counts**		
1	16	21	29	22.00	6.56	-6.56
2	100	115	133	116.00	16.52	-16.52
3	268	217	229	238.00	26.66	-26.66
4	99	86	100	95.00	7.81	-7.81
5	60	55	59	58.00	2.65	-2.65
6	30	40	40	36.67	5.77	-5.77
7	21	32	29	27.33	5.69	-5.69
8	11	20	19	16.67	4.93	-4.93
9	3	10	8	7.00	3.61	-3.61
10	0	2	0	0.67	1.15	-1.15
11	0	0	0	0.00	0.00	0.00
Isolates : Cervical neoplasia (CN) 6						
No of trophozoites x 10^4 *T. vaginalis*/ml					Standard Deviation (±)	
Days	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average counts**		
1	25	16	30	23.67	7.09	-7.09
2	77	53	73	67.67	12.86	-12.86
3	221	218	239	226.00	11.36	-11.36
4	89	98	91	92.67	4.73	-4.73
5	36	32	33	33.67	2.08	-2.08
6	28	25	28	27.00	1.73	-1.73
7	17	18	16	17.00	1.00	-1.00
8	11	10	11	10.67	0.58	-0.58
9	2	2	1	1.67	0.58	-0.58
10	0	0	0	0.00	0.00	0.00
11	0	0	0	0.00	0.00	0.00

Appendix E

Statistical analysis results for average trophozoite counts between days of CN isolates (CN 1- CN 6) in 10^4 *T. vaginalis*/ml

1. One-Way ANOVA was performed to test the equality of variances between different days of trophozoite observation

Table 2: ANOVA table

Test of Homogeneity of Variances

Average trophozoites count in 10^4 *T. vaginalis*/ml

Levene Statistic	df1	df2	Sig.
7.586	10	55	.000

Test Statistics[a,b]

	Average trophozoites count in 10^4 *T. vaginalis*/ml
Chi-Square	62.630
df	10
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: Days of count

Output:

a. Test of homogeneity of variances: $p < 0.05$, equality of variances not assumed
b. Kruskal-Wallis: p-value is 0.000, which is less than 0.05. Hence, at least one pair of means between the days differs significantly

Hence, to identify the pairs that differ significantly, post-hoc test was performed using Dunnett T3 since equality of variances cannot be assumed.

Table 3: Statistical comparison between days for average trophozoite counts of CN isolates in 10^4 *T. vaginalis*/ml

Multiple Comparisons						
Dependent Variable: Average trophozoites count in 10^4 *T. vaginalis*/ml Dunnett T3						
(I) Days of count	(J) Days of count	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Day 0	Day 1	-22.16833	4.29572	.065	-45.8787	1.5420
	Day 2	-101.66833*	10.75371	.005	-161.0237	-42.3129
	Day 3	-223.22333*	5.36184	.000	-252.8181	-193.6285
	Day 4	-114.00000*	14.51240	.011	-194.1016	-33.8984
	Day 5	-61.83500*	6.52545	.005	-97.8524	-25.8176
	Day 6	-34.00167*	4.71778	.016	-60.0416	-7.9617
	Day 7	-20.72000*	2.00254	.003	-31.7731	-9.6669
	Day 8	-11.89000*	1.20969	.004	-18.5669	-5.2131
	Day 9	-3.16667	1.03100	.371	-8.8573	2.5240
	Day 10	.16667	.39196	1.000	-1.9968	2.3301
Day 1	Day 0	22.16833	4.29572	.065	-1.5420	45.8787
	Day 2	-79.50000*	11.57996	.009	-136.4845	-22.5155
	Day 3	-201.05500*	6.87041	.000	-231.1048	-171.0052
	Day 4	-91.83167*	15.13483	.023	-169.6081	-14.0553
	Day 5	-39.66667*	7.81247	.024	-74.7762	-4.5571
	Day 6	-11.83333	6.38049	.898	-39.4697	15.8030
	Day 7	1.44833	4.73956	1.000	-21.2459	24.1425
	Day 8	10.27833	4.46280	.672	-12.7890	33.3457
	Day 9	19.00167	4.41771	.115	-4.2016	42.2049
	Day 10	22.33500	4.31357	.063	-1.2871	45.9571
Day 2	Day 0	101.66833*	10.75371	.005	42.3129	161.0237
	Day 1	79.50000*	11.57996	.009	22.5155	136.4845
	Day 3	-121.55500*	12.01630	.000	-178.3735	-64.7365
	Day 4	-12.33167	18.06245	1.000	-92.0708	67.4075
	Day 5	39.83333	12.57870	.276	-17.5001	97.1667
	Day 6	67.66667*	11.74307	.020	10.8127	124.5206

	Day 7	80.94833*	10.93857	.012	22.4262	139.4704
	Day 8	89.77833*	10.82153	.008	30.7533	148.8033
	Day 9	98.50167*	10.80302	.005	39.3894	157.6139
	Day 10	101.83500*	10.76085	.005	42.5158	161.1542
Day 3	Day 0	223.22333*	5.36184	.000	193.6285	252.8181
	Day 1	201.05500*	6.87041	.000	171.0052	231.1048
	Day 2	121.55500*	12.01630	.000	64.7365	178.3735
	Day 4	109.22333*	15.47124	.008	32.1207	186.3260
	Day 5	161.38833*	8.44575	.000	124.5406	198.2361
	Day 6	189.22167*	7.14190	.000	158.2293	220.2140
	Day 7	202.50333*	5.72359	.000	174.0276	230.9791
	Day 8	211.33333*	5.49660	.000	182.3122	240.3545
	Day 9	220.05667*	5.46006	.000	190.9010	249.2123
	Day 10	223.39000*	5.37614	.000	193.8667	252.9133
Day 4	Day 0	114.00000*	14.51240	.011	33.8984	194.1016
	Day 1	91.83167*	15.13483	.023	14.0553	169.6081
	Day 2	12.33167	18.06245	1.000	-67.4075	92.0708
	Day 3	-109.22333*	15.47124	.008	-186.3260	-32.1207
	Day 5	52.16500	15.91199	.265	-24.5312	128.8612
	Day 6	79.99833*	15.25999	.043	2.5138	157.4829
	Day 7	93.28000*	14.64992	.025	13.8333	172.7267
	Day 8	102.11000*	14.56273	.017	22.2585	181.9615
	Day 9	110.83333*	14.54898	.012	30.9147	190.7519
	Day 10	114.16667*	14.51770	.011	34.0919	194.2414
Day 5	Day 0	61.83500*	6.52545	.005	25.8176	97.8524
	Day 1	39.66667*	7.81247	.024	4.5571	74.7762
	Day 2	-39.83333	12.57870	.276	-97.1667	17.5001
	Day 3	-161.38833*	8.44575	.000	-198.2361	-124.5406
	Day 4	-52.16500	15.91199	.265	-128.8612	24.5312
	Day 6	27.83333	8.05226	.182	-7.8347	63.5013
	Day 7	41.11500*	6.82581	.023	6.1936	76.0364
	Day 8	49.94500*	6.63663	.011	14.4293	85.4607
	Day 9	58.66833*	6.60639	.005	23.0285	94.3081
	Day 10	62.00167*	6.53721	.004	26.0434	97.9599
Day 6	Day 0	34.00167*	4.71778	.016	7.9617	60.0416

	Day 1	11.83333	6.38049	.898	-15.8030	39.4697
	Day 2	-67.66667*	11.74307	.020	-124.5206	-10.8127
	Day 3	-189.22167*	7.14190	.000	-220.2140	-158.2293
	Day 4	-79.99833*	15.25999	.043	-157.4829	-2.5138
	Day 5	-27.83333	8.05226	.182	-63.5013	7.8347
	Day 7	13.28167	5.12520	.529	-11.6777	38.2410
	Day 8	22.11167	4.87040	.091	-3.3119	47.5352
	Day 9	30.83500*	4.82912	.021	5.2743	56.3957
	Day 10	34.16833*	4.73404	.015	8.2092	60.1275
Day 7	Day 0	20.72000*	2.00254	.003	9.6669	31.7731
	Day 1	-1.44833	4.73956	1.000	-24.1425	21.2459
	Day 2	-80.94833*	10.93857	.012	-139.4704	-22.4262
	Day 3	-202.50333*	5.72359	.000	-230.9791	-174.0276
	Day 4	-93.28000*	14.64992	.025	-172.7267	-13.8333
	Day 5	-41.11500*	6.82581	.023	-76.0364	-6.1936
	Day 6	-13.28167	5.12520	.529	-38.2410	11.6777
	Day 8	8.83000	2.33956	.132	-1.8423	19.5023
	Day 9	17.55333*	2.25236	.002	6.9675	28.1392
	Day 10	20.88667*	2.04054	.002	10.0027	31.7707
Day 8	Day 0	11.89000*	1.20969	.004	5.2131	18.5669
	Day 1	-10.27833	4.46280	.672	-33.3457	12.7890
	Day 2	-89.77833*	10.82153	.008	-148.8033	-30.7533
	Day 3	-211.33333*	5.49660	.000	-240.3545	-182.3122
	Day 4	-102.11000*	14.56273	.017	-181.9615	-22.2585
	Day 5	-49.94500*	6.63663	.011	-85.4607	-14.4293
	Day 6	-22.11167	4.87040	.091	-47.5352	3.3119
	Day 7	-8.83000	2.33956	.132	-19.5023	1.8423
	Day 9	8.72333*	1.58944	.011	1.8105	15.6362
	Day 10	12.05667*	1.27161	.002	5.5970	18.5163
Day 9	Day 0	3.16667	1.03100	.371	-2.5240	8.8573
	Day 1	-19.00167	4.41771	.115	-42.2049	4.2016
	Day 2	-98.50167*	10.80302	.005	-157.6139	-39.3894
	Day 3	-220.05667*	5.46006	.000	-249.2123	-190.9010
	Day 4	-110.83333*	14.54898	.012	-190.7519	-30.9147
	Day 5	-58.66833*	6.60639	.005	-94.3081	-23.0285

	Day 6	-30.83500*	4.82912	.021	-56.3957	-5.2743
	Day 7	-17.55333*	2.25236	.002	-28.1392	-6.9675
	Day 8	-8.72333*	1.58944	.011	-15.6362	-1.8105
	Day 10	3.33333	1.10299	.356	-2.1387	8.8054
Day 10	Day 0	-.16667	.39196	1.000	-2.3301	1.9968
	Day 1	-22.33500	4.31357	.063	-45.9571	1.2871
	Day 2	-101.83500*	10.76085	.005	-161.1542	-42.5158
	Day 3	-223.39000*	5.37614	.000	-252.9133	-193.8667
	Day 4	-114.16667*	14.51770	.011	-194.2414	-34.0919
	Day 5	-62.00167*	6.53721	.004	-97.9599	-26.0434
	Day 6	-34.16833*	4.73404	.015	-60.1275	-8.2092
	Day 7	-20.88667*	2.04054	.002	-31.7707	-10.0027
	Day 8	-12.05667*	1.27161	.002	-18.5163	-5.5970
	Day 9	-3.33333	1.10299	.356	-8.8054	2.1387
*. The mean difference is significant at the 0.05 level.						

Output:

a. The mean differences of average trophozoites for day 3 with other days were positive and p-value is less than 0.05, hence day three marked a significantly different trophozoite count.

Appendix F

Raw data of trophozoite count in CN isolates of *T. vaginalis* for concentration of 10^5 *T. vaginalis*/ml

Isolates : Cervical neoplasia (CN) 1						
Days	**No of trophozoites x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	50	68	70	62.67	11.02	-11.02
2	122	135	106	121.00	14.53	-14.53
3	333	397	355	361.67	32.52	-32.52
4	245	229	237	237.00	8.00	-8.00
5	77	75	44	65.33	18.50	-18.50
6	3	4	2	3.00	1.00	-1.00
7	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of trophozoites x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	39	19	37	31.67	11.02	-11.02
2	156	166	128	150.00	19.70	-19.70
3	390	400	321	370.33	43.02	-43.02
4	200	222	242	221.33	21.01	-21.01
5	32	49	21	34.00	14.11	-14.11
6	4	5	10	6.33	3.21	-3.21
7	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 3						
Days	**No of trophozoites x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	39	46	39	41.33	4.04	-4.04
2	153	154	112	139.67	23.97	-23.97
3	453	500	578	510.33	63.14	-63.14
4	287	249	266	267.33	19.04	-19.04
5	45	40	38	41.00	3.61	-3.61
6	21	11	9	13.67	6.43	-6.43
7	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of trophozoites x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	89	77	70	78.67	9.61	-9.61
2	143	133	149	141.67	8.08	-8.08
3	375	412	477	421.33	51.64	-51.64
4	251	222	210	227.67	21.08	-21.08
5	100	98	75	91.00	13.89	-13.89
6	13	25	33	23.67	10.07	-10.07
7	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
Days	**No of trophozoites x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	30	36	39	35.00	4.58	-4.58
2	175	177	151	167.67	14.47	-14.47
3	399	380	418	399.00	19.00	-19.00
4	205	237	212	218.00	16.82	-16.82
5	78	37	45	53.33	21.73	-21.73
6	1	0	0	0.33	0.58	-0.58
7	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of trophozoites x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	35	49	69	51.00	17.09	-17.09
2	103	124	155	127.33	26.16	-26.16
3	521	498	519	512.67	12.74	-12.74
4	259	230	203	230.67	28.01	-28.01
5	111	121	97	109.67	12.06	-12.06
6	40	37	23	33.33	9.07	-9.07
7	0	0	0	0.00	0.00	0.00

Appendix G

1. Raw data on average mean of amoeboid form in six CN isolates (CN1-CN6) at 10^5 *T. vaginalis*/ml (overcrowding of parasites).

Isolates : Cervical neoplasia (CN) 1						
Days	**No of amoeboid x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	1	1	2	1.33	0.58	-0.58
2	3	3	3	3.00	0.00	0.00
3	10	4	8	7.33	3.06	-3.06
4	1	2	2	1.67	0.58	-0.58
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of amoeboid x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	1	4	1	2.00	1.73	-1.73
2	5	5	6	5.33	0.58	-0.58
3	11	20	12	14.33	4.93	-4.93
4	1	2	1	1.33	0.58	-0.58
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 3						
Days	**No of amoeboid x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	1	2	1	1.33	0.58	-0.58
2	7	8	6	7.00	1.00	-1.00
3	10	5	8	7.67	2.52	-2.52
4	0	0	1	0.33	0.58	-0.58
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of amoeboid x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	3	5	2	3.33	1.53	-1.53
2	11	10	8	9.67	1.53	-1.53
3	15	15	9	13.00	3.46	-3.46
4	3	2	0	1.67	1.53	-1.53
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	1	4	4	3.00	1.73	-1.73
2	3	9	10	7.33	3.79	-3.79
3	5	11	12	9.33	3.79	-3.79
4	1	1	3	1.67	1.15	-1.15
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of amoeboid x 10^5 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	2	2	2	2.00	0.00	0.00
2	3	5	5	4.33	1.15	-1.15
3	7	7	9	7.67	1.15	-1.15
4	1	2	0	1.00	1.00	-1.00
5	0	0	0	0.00	0.00	0.00

Appendix H

Raw data on average mean of amoeboid forms in six CN isolates (CN1-CN6) for different horse serum concentrations.

1. 10 % horse serum concentrations

Horse serum concentrations : 10%						
Isolates : Cervical neoplasia (CN) 1						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	3	2	2	2.33	0.58	-0.58
3	3	3	2	2.67	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0
2	3	1	2	2.00	1.00	-1.00
3	3	1	3	2.33	1.15	- 1.15
4	0	0	0	0.00	0.00	0
5	0	0	0	0.00	0.00	0

Isolates : Cervical neoplasia (CN) 3						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	1	2	1.33	0.58	-0.58
3	3	2	2	2.33	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	0	0	1	0.33	0.58	-0.58
3	1	2	2	1.67	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	1	0	0.33	0.58	-0.58
2	1	2	0	1.00	1.00	-1.00
3	3	3	3	3.00	0.00	0.00
4	0	1	0	0.33	0.58	-0.58
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	0	2	0	0.67	1.15	-1.15
3	3	4	6	4.33	1.53	-1.53
4	1	2	0	1.00	1.00	-1.00
5	0	0	0	0.00	0.00	0.00

2. 5% horse serum concentrations

Horse serum concentrations : 5%						
Isolates : Cervical neoplasia (CN) 1						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	0	1	0	0.33	0.58	-0.58
3	4	2	3	3.00	1.00	-1.00
4	1	1	0	0.67	0.58	-0.58
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	0	0	0.33	0.58	-0.58
3	2	2	2	2.00	0.00	0.00
4	1	0	1	0.67	0.58	-0.58
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 3						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	1	0	0	0.33	0.58	-0.58
2	1	1	1	1.00	0.00	0.00
3	3	2	2	2.33	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	2	0	1.00	1.00	-1.00
3	2	2	2	2.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	1	0	0.33	0.58	-0.58
2	0	2	0	0.67	1.15	-1.15
3	3	3	3	3.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	1	0	0.33	0.58	-0.58
2	1	2	0	1.00	1.00	-1.00
3	2	5	6	4.33	2.08	-2.08
4	1	0	0	0.33	0.58	-0.58
5	0	0	0	0.00	0.00	0.00

3. 15 % horse serum concentrations

Horse serum concentrations : 15%						
Isolates : Cervical neoplasia (CN) 1						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	3	1	0	1.33	1.53	-1.53
2	2	9	2	4.33	4.04	-4.04
3	6	7	3	5.33	2.08	-2.08
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	2	1	0	1.00	1.00	-1.00
2	7	4	2	4.33	2.52	-2.52
3	1	3	2	2.00	1.00	-1.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 3						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	1	0	0.33	0.58	-0.58
2	3	5	7	5.00	2.00	-2.00
3	1	3	2	2.00	1.00	-1.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	1	0	0	0.33	0.58	-0.58
2	2	4	6	4.00	2.00	-2.00
3	4	6	3	4.33	1.53	-1.53
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	10	5	6	7.00	2.65	-2.65
3	6	8	9	7.67	1.53	-1.53
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	6	8	9	7.67	1.53	-1.53
3	5	3	1	3.00	2.00	-2.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

4. Raw data on average mean of amoeboid form in six CN isolates (CN1-CN6) for 20 % horse serum concentrations

Horse serum concentrations : 20%						
Isolates : Cervical neoplasia (CN) 1						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	2	5	3	3.33	1.53	-1.53
3	2	3	2	2.33	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	3	8	5	5.33	2.52	-2.52
3	3	3	2	2.67	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 3						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	2	4	5	3.67	1.53	-1.53
3	3	3	2	2.67	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	5	2	4	3.67	1.53	-1.53
3	2	1	3	2.00	1.00	-1.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00
Isolates : Cervical neoplasia (CN) 5						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	5	5	3	4.33	1.15	-1.15
3	3	4	5	4.00	1.00	-1.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	2	2	2	2.00	0.00	0.00
3	3	2	1	2.00	1.00	-1.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Appendix I

Raw data on average mean of amoeboid forms in six CN isolates (CN1-CN6) for different concentrations of metronidazole drug.

1. 0.001 mg/ml of metronidazole drug

Metro drug : 0.001 mg/ml						
Isolates : Cervical neoplasia (CN) 1						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	2	1	2	1.67	0.58	-0.58
3	0	0	0	0.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	2	2	1	1.67	0.58	-0.58
3	0	0	0	0.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 3						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	2	2	3	2.33	0.58	-0.58
3	0	0	0	0.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	**0**	0	0	0.00	0.00	0.00
2	2	2	1	1.67	0.58	-0.58
3	0	0	0	0.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	0	2	1.00	1.00	-1.00
3	0	0	0	0.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	1	1	1.00	0.00	0.00
3	0	0	0	0.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

2. 0.0001 mg/ml of metronidazole drug

Metro drug : 0.0001 mg/ml						
Isolates : Cervical neoplasia (CN) 1						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	2	1	1.33	0.58	-0.58
3	2	2	2	2.00	0.00	0.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 2						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	
2	2	3	2	2.33	0.58	
3	3	1	2	2.00	1.00	
4	0	0	0	0.00	0.00	
5	0	0	0	0.00	0.00	

Isolates : Cervical neoplasia (CN) 3						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	1	2	1.33	0.58	-0.58
3	4	1	2	2.33	1.53	-1.53
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 4						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	0	1	0.67	0.58	-0.58
3	2	2	4	2.67	1.15	-1.15
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 5						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	1	1	1	1.00	0.00	0.00
3	3	3	2	2.67	0.58	-0.58
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Isolates : Cervical neoplasia (CN) 6						
Days	**No of amoeboid x 10^4 *T. vaginalis*/ml**				**Standard Deviation (±)**	
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average counts**		
1	0	0	0	0.00	0.00	0.00
2	0	1	0	0.33	0.58	-0.58
3	5	3	1	3.00	2.00	-2.00
4	0	0	0	0.00	0.00	0.00
5	0	0	0	0.00	0.00	0.00

Control	**No of amoeboid x 10^4 *T. vaginalis*/ml**					**Average counts**
	DAY1	**DAY 2**	**DAY 3**	**DAY 4**	**DAY 5**	
CN1	0	2	3	1	0	6.00 ± 1.30
CN2	0	3	3	1	0	7.00 ± 1.52
CN3	0	2	2	1	0	5.00 ± 1.00
CN4	0	3	2	0	0	5.00 ± 1.41
CN5	0	3	2	0	0	5.00 ± 1.41
CN6	0	2	3	1	0	6.00 ± 1.30

Appendix J

Statistical analysis of average amoeboid forms in CN isolates of *T. vaginalis* between 10^4 *T. vaginalis*/ml and 10^5 *T. vaginalis*/ml

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Day 1	Equal variances assumed	9.512	.012	-5.893	10	**.000**	-2.05500	.34874	-2.83204	-1.27796
	Equal variances not assumed			-5.893	5.414	.002	-2.05500	.34874	-2.93124	-1.17876
Day 2	Equal variances assumed	12.347	.006	-5.280	10	**.000**	-5.16500	.97829	-7.34477	-2.98523
	Equal variances not assumed			-5.280	5.109	.003	-5.16500	.97829	-7.66378	-2.66622
Day 3	Equal variances assumed	13.950	.004	-5.256	10	**.000**	-6.66500	1.26815	-9.49061	-3.83939
	Equal variances not assumed			-5.256	5.462	.003	-6.66500	1.26815	-9.84361	-3.48639
Day 4	Equal variances assumed	1.930	.195	-3.155	10	.010	-.78000	.24726	-1.33094	-.22906
	Equal variances not assumed			-3.155	7.549	**.015**	-.78000	.24726	-1.35618	-.20382

Output: p-value is less than 0.05 (bold), thus there is a significant difference in average amoeboid count between days for the two cell concentrations. Average amoeboid forms in 10^5 *T. vaginalis*/ml is higher than in 10^4 *T. vaginalis*/ml in day 1 to day 4.

Appendix K

Statistical analysis between different concentrations of horse serum for amoeboid forms in CN isolates (CN1-CN6) using Dunnett T3.

Multiple Comparisons

Dependent Variable: Average amoeboid count

Dunnett T3

(I) Different horse serum concentrations	(J) Different horse serum concentrations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1%	5%	-3.94500*	.45058	**.002**	-5.8878	-2.0022
	10%	-4.33333*	.43016	**.001**	-6.1880	-2.4786
	15%	-9.94500*	1.14406	**.002**	-14.8778	-5.0122
	20%	-6.33333*	.66053	**.001**	-9.1813	-3.4854
5%	1%	3.94500*	.45058	**.002**	2.0022	5.8878
	10%	-.38833	.62295	.999	-2.5493	1.7727
	15%	-6.00000*	1.22959	**.017**	-10.7881	-1.2119
	20%	-2.38833	.79958	**.118**	-5.2417	.4651
10%	1%	4.33333*	.43016	**.001**	2.4786	6.1880
	5%	.38833	.62295	.999	-1.7727	2.5493
	15%	-5.61167*	1.22226	**.024**	-10.4034	-.8200
	20%	-2.00000	.78825	**.227**	-4.8315	.8315
15%	1%	9.94500*	1.14406	**.002**	5.0122	14.8778
	5%	6.00000*	1.22959	**.017**	1.2119	10.7881
	10%	5.61167*	1.22226	**.024**	.8200	10.4034
	20%	3.61167	1.32105	.180	-1.2235	8.4468
20%	1%	6.33333*	.66053	**.001**	3.4854	9.1813
	5%	2.38833	.79958	.118	-.4651	5.2417
	10%	2.00000	.78825	.227	-.8315	4.8315
	15%	-3.61167	1.32105	.180	-8.4468	1.2235

*. The mean difference is significant at the 0.05 level.

Appendix L

Statistical analysis between different concentrations of metronidazole drug for amoeboid forms in CN isolates (CN1-CN6) using Tukey HSD.

Multiple Comparisons

Dependent Variable: Average count
Tukey HSD

(I) Effect of metronidazole drug	(J) Effect of metronidazole drug	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	0.001	7.11000*	.40416	.000	6.0602	8.1598
	0.0001	5.05667*	.40416	.000	4.0069	6.1065
0.001	Control	-7.11000*	.40416	.000	-8.1598	-6.0602
	0.0001	-2.05333*	.40416	.000	-3.1031	-1.0035
0.0001	Control	-5.05667*	.40416	.000	-6.1065	-4.0069
	0.001	2.05333*	.40416	.000	1.0035	3.1031

*. The mean difference is significant at the 0.05 level.

Output:

p-value is less than 0.05. Hence there is a significant difference of average amoeboid forms between different concentrations of metronidazole drug with significantly higher count in control. Between the two concentrations, average amoeboid forms in 0.0001 mg/ml were significantly higher than in 0.001 mg/ml.