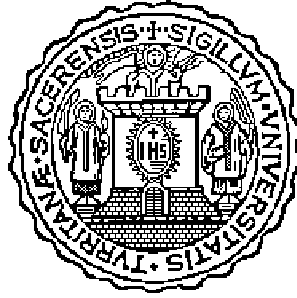

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PREVALENCE OF TRICHOMONIASIS IN HUE CITY, VIETNAM: A SEROLOGICAL STUDY

Direttore:
Professor Bruno Masala

Tutor:
Professor Pier Luigi Fiori

Tesi di dottorato della:
Dott.ssa Ton Nu Phuong Anh

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LIST OF ABBREVIATION

AUC	Area under the curve
bp	base pairs
BSA	Bovin serum albumin
BV	Bacterial vaginosis
CDC	Center for Disease Control and Prevention
CI	Confidence interval
CP	Cysteine proteinases
CSW	Commercial sex worker
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drugs Administration
FISH	Fluorescent in situ hybridization
HIV	Human immunodeficiency virus
HVEC	Human vaginal epithelial cells
LF	Lytic factor
LPG	Lipophosphoglycan
MAb	Monoclonal antibody
Mh	*Mycoplasma hominis*
MW	Molecular weigh
NC	Negative control
NO	Nitric oxide
No	Number
OD	Optical density
Pa	Patient

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT	Room temperature
SD	Standard deviation
SDS- PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STI	Sexually transmitted infection
STD	Sexually transmitted disease
Tv	*Trichomonas vaginalis*
Uu	*Ureplasma urealyticum*
vs.	versus
VWs	Vaginal washes
WB	Western blot
WHO	World Health Organisation

ABSTRACT

The protist *Trichomonas vaginalis* is the most common non-viral, curable, sexually transmitted disease (STD) agent worldwide. The overall objective of this study is to determine the prevalence of trichomoniasis patients in Hue City, Vietnam and its serological patterns.

The study included 249 symptomatic women, 534 asymptomatic women, 38 healthy men, and 50 sera of children 2-10 years of age. In addition, specific anti - *T. vaginalis* antibody response was studied in a group of 46 women affected by trichomoniasis and 8 male sexual partners. All women were subjected to standard clinical examination and vaginal samples were collected for identification of *Trichomonas vaginalis* by wet mount and cultivation in specific media. Sera from patients were used to set up immunoenzymatic techniques to detect specific antibody response for seroepidemiological studies.

In addition, serological reactivity of patients affected by trichomoniasis was studied for a 5 months period after pharmacological treatment in order to estimate persistence of anti-trichomonas antibodies after eradication of protozoan infection. Multiplex polymerase chain reaction (PCR) has been used for *T. vaginalis*, *M. hominis*, *U. urealyticum* testing in pretreatment time and every month during five months follow-up.

The prevalence of trichomoniasis diagnosed by microscopic examination in symptomatic women and asymptomatic groups were 19.3% (42/243, 95% CI = 12.8% - 22.7%) and 0.7% (4/534, 95% CI = 0.18% - 1.8%), respectively.

The most prevalent symptoms were vaginal erythema (80.4%), malodorous vaginal discharge (73.9%), profuse vaginal discharge (60.9%), cervicitis (58.7%), and yellowish-green frothy discharge (54.3%), and 10.9% asymptomatic patients. There were 4 (8.7%) menopausal patients. A mixed

infection, namely co-infection with *M. hominis, U. urealyticum* and both of them has been recorded in 39.1%, 23.9% and 28.3%, respectively. There were only 8.7% infected *T. vaginalis* alone.

ELISA assay using the whole cell of *T. vaginalis* for detection of the human sera IgG antibody yielded high sensitivity and specificity (93.48% and 84.88%, respectively).

The seroprevalence from general population were found 18.9% in women and 8.7% in men. The seroprevalence were 31.3% in symptomatic women, 13.3% in asymptomatic women. The seroprevalence was 14% in safe sex behavior women to compare with 22.7% in unsafe sex behavior women. There were 7.9% seropositive from sera of healthy men and 12.5% seropositive from sera of men partners of trichomoniasis women.

Serological follow-up by ELISA showed the trending line of sera *T. vaginalis* IgG antibody going down after 4-5 months in the group of recovered patients; while those from the unrecovered/re-infection patients kept the high level of IgG antibody, a marker of infection persistence. Results from Western blot analysis showed very good correlation with those from ELISA assay and clinical symptoms during the course of follow-up periods. There were the persistence of antibody to *T. vaginalis* antigen 84 -115kDa in men partners and recovered patients.

In general, the prevalence of *T. vaginalis* infection is high in symptomatic women and low in asymptomatic women. Clinical pattern showed a wide spectrum of appearance, from asymptomatic women to typical infectious symptoms. ELISA essay yielded high sensitivity and specificity. The variation of 5-month follow-up sera *T. vaginalis* IgG antibody was different between the groups of recovered and unrecovered/re-infection patients.

1. INTRODUCTION

1.1. *Trichomonas vaginalis*

1.1.1. The history of *Trichomonas vaginalis*

Trichomonas vaginalis (*T. vaginalis*) is a extracellular single-cell, flagellated parasite that usually lives in the female lower reproductive tract and rarely the male urethra, classified as a sexually transmitted agent[1,2,3]. Unique genetic and structural features place the parasite at the base of the eukaryotic phylogenetic tree and suggest an intriguing evolution toward mucosal parasitism.

T. vaginalis (*T.v*) was first recorded in the medical literature more than 170 years ago by a French physician, Alfred Donné, who described the trichomonad in human vaginal discharge in 1836. Then, Hohne (1916), Wendberger (1936), and Jirovec (1942) asserted that *T. vaginalis* was the etiological agent in some cases of vaginitis. That concept took many years to become universally accepted[4,5].

1.1.2. Morphology and structure

T. vaginalis is the largest of the human trichomonads, a primitive eukaryotic organism with most respects similar to other eukaroyotes. However its energy metabolism is similar to that of anaerobic bacteria. The organism has no cyst form[6], but recent researchs have suggested that under unfavorable conditions they may assume a pseudocyst form[7, 8].

Light microscopy (Figure 1. 1) shows living *T. vaginalis* to be pear-shaped, approximately 10-13x8-10µm. Fixed and stained organisms are about 25% smaller. They have four anterior flagella and a fold of cytoplasm, the running undulating membrane, along one side of the body for about two-thirds of its length. The latter is supported by a third rod call the costa; its wave-like motion is produced by a fifth flagellum attached to it [5].

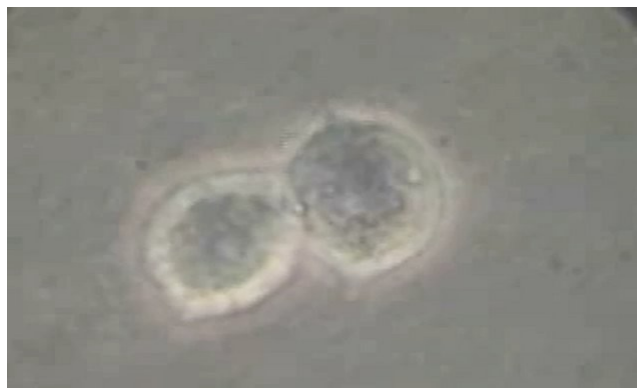


Figure 1.1. *T. vaginalis* on light microscopy.

In *T. vaginalis* and *T. tenax* this does not extend beyond the end of the undulating membrane to form a free flagellum, while *T. hominis* does. A rigid microtubular rod - called the axostyle - run through the body and appears to project from its posterior end; the prominent nucleus is enfolded by the anterior end of the axostyle[9]. (Figure 1. 2)

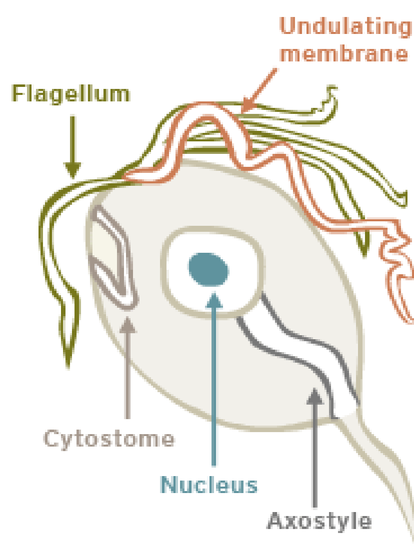


Figure 1.2. Morphology of T. vaginalis

On electron microscopy (Figure 1. 3), the deeply staining parabasal body to consist of an elaborate Golgi complex supported by filaments, anterior to this basal bodies (one orthogonal to the other four), from which the flagella arise, comprise the kinetosomal complex. The shape of the organism is maintained by an intricate system of microtubular organelles. A considerable number of

electron-dense granules are also presents arranged alongside the costa and the axostyle; these are now identified as hydrogenosomes[10].

The above-description applied to *T.vaginalis* in clinical specimens or free in culture, but it will adhere to cultured cells and some non-living surface, it becoming much more amoeboid. When it comes to contact with vaginal epithelial cell in vitro, the organism become flattened and adherent [11].

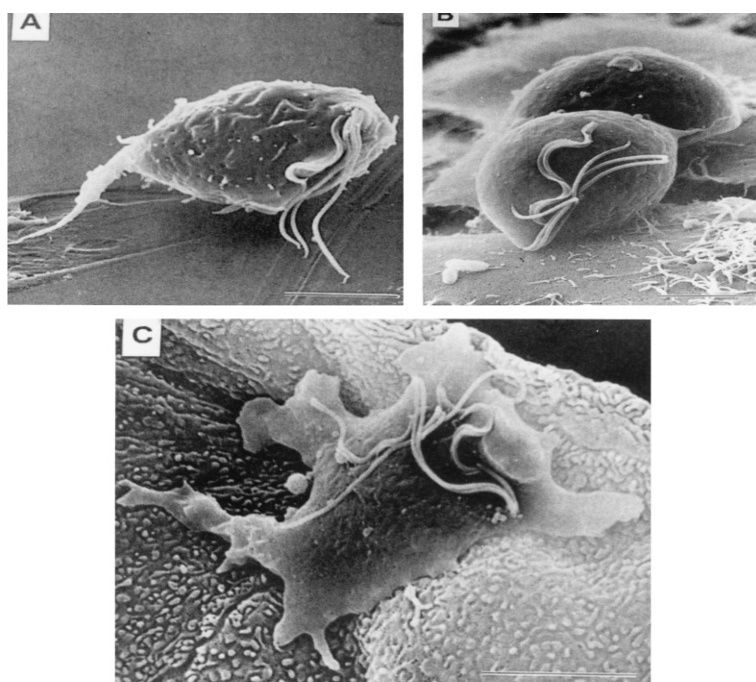


Figure. 1.3. (A). *T. vaginalis* in broth culture: the axostyle, undulating membrane, and flagella. (B) *T. vaginalis* on the surface of a vaginal epithelial cell prior to amoeboid transformation. (C) Amoeboid morphology of *T. vaginalis* in cell culture[11] (Arroyo R et al. 1993).

The first draft sequence of *T. vaginalis*'s genome was published in January 2007. According to the data from this publication, *T. vaginalis*'s genome is among the largest on record - approximately the size of the human genome - containing a large number of repeated or transposable genes[12].

1.1.3. Classification

T. vaginalis is a parasitic protozoan, and the taxonomic position is based on classification scheme by Dyer, belongs to the family *Trichomonadidae* (Wenyon, 1926), having a cytostome, three to five free flagella (one flagellum on the margin of the undulating membrane); axostyle protruding through the posterior of the cell; genus *Trichomonas*: four free flagella; one recurrent, along the outer margin of the undulating membrane; a costa at the base of the undulating membrane, and an axostyle extending through the cell and species of *Trichomonas vaginalis* (Donne´, 1836)[13].

1.2. Epidemiology

Trichomonal infection has been encountered and recognized in every continent, every climate region and with no seasonal variability. It has a cosmopolitan distribution and has been identified in all racial groups and socioeconomic strata. According to data from WHO (1995), the estimated incidence is more than 170 million cases worldwide[14], at least 2 to 3 million symptomatic infections occur annually among sexually active women in the United States (1995). But in the year of 2000, there were over 180 million people worldwide, including 8 to 10 million Americans, become infected with *T. vaginalis* annually[15].

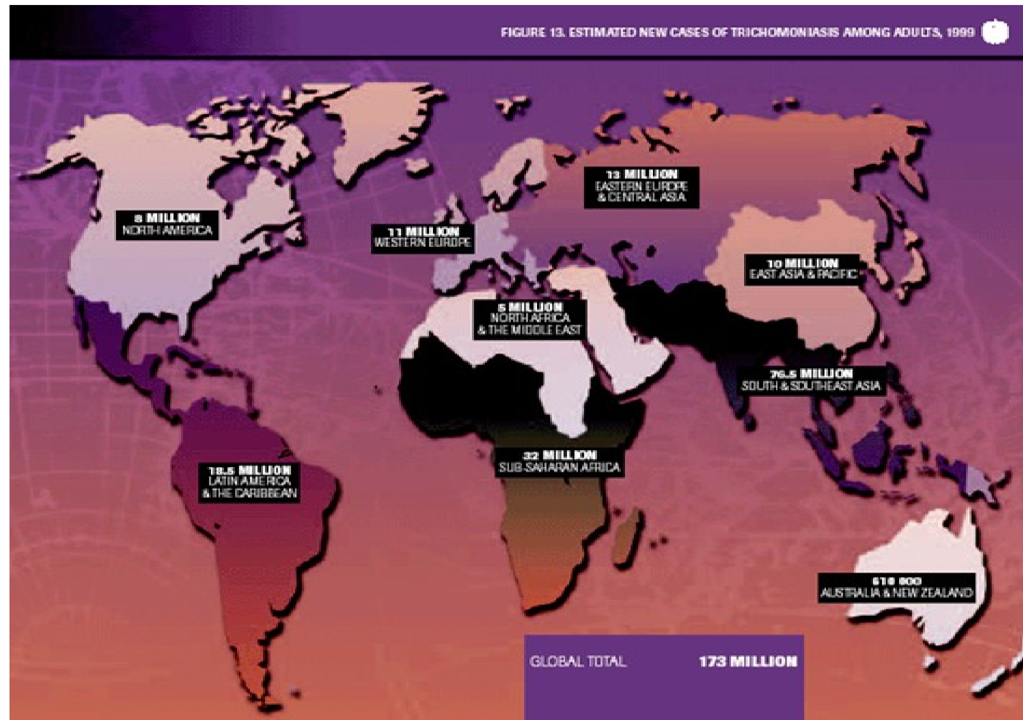


Figure 1.4. The estimated new cases of Trichomoniasis among adults (WHO, 1999)[16].

The prevalence *T. vaginalis* of among users of a primary health care clinic in São Paulo, Brasil was 3.2% in 2011[17]. The overall prevalence of *T. vaginalis* in the general population in Flanders was 0.37%[18]. In the period January-June 2006, Trevisan investigated 207 subjects at the Microbiology and Virology Service of Padua's Hospital, Italia in 18-65 years old, males and women, Italian and foreigners. The prevalence of *T. vaginalis* was 3.86%[19]. The prevalence of *Trichomonas vaginalis* infection was based on a large cross-sectional survey conducted in 2004-2005 among randomly sampled women (18-45 years) from the computerized population registries in Denmark, Iceland, Norway, and Sweden was 1.5%[20].

The prevalence of *T. vaginalis* infections in rural sub-Saharan Africa was 31% in 2010[21]. According to Dunne (2003), the prevalence of *Trichomonas*

vaginalis in some country were: 67% in Mongolia, 40-60% in Africa, 40% in Indigenous Australians, 46% in highland women of Papua New Guinea[22] .

The prevalence of *Trichomonas vaginalis* infection in married women aged 25-54 years in Beijing, China (2011) was 1%[23].The prevalence of *T. vaginalis* infection in women sex workers in Thailand (2000) was 1%[24].

In Vietnam, the study of Lan PT et al. (2008) showed that the prevalence of *Trichomonas vaginalis* infection in general pupolation of Bavi - Hanoi, was 1%[25], and according to Anh PK et al in Hanoi, Vietnam (1998) was 1.3%[26], in South of Vietnam (Soc Trang) 8.9%[27]. In addition we didn't find any reports of metronidazole resistance in Vietnam.

The trichomoniasis prevalence depends on many factors including age, sexual activity, number of sexual partners, other STDs, sexual customs, phase of the menstrual cycle, techniques of examination, specimen collection, and laboratory techniques. Since the disease showed a wide spectrum of clinical pattern, in order to be controlled effectively, its requires the screening of women and their partners and the appropriate treatment of infected individuals.

1.3. Pathogenicity of *Trichomonas vaginalis*

The *T. vaginalis* virulence factors response remain elusive (Singh B. N. et al., 2009)[28]. It seems likely that the severity of the trichomoniasis in women is due to both host and parasites-related factors. It was known that about one-third of untreated asymptomatic become symptomatic over the following 6 months [6].

Using light microscopy and scanning electron microscopy, Heath J.P. (1981) performed study on the behavior and pathogenic effects of *Trichomonas vaginalis* in mammalian cell cultures and found that they can destroy epithelial monolayer cells after inoculation of the parasites into the cell cultures. After adhering to the vaginal epithelial cells, the parasites changed

an amoeboid morphology, and then crawled over and under the monolayer of epithelial cells cells. Its amoeboid morphology, its adhesive capacity and the motility may be important mechanisms causing vaginal epithelium lesion[29].

Many experimentally determined activities of the parasites that have been suggested as virulence factors include their abilities involved in adhesion, proteolytis, haemolysis, detachment of cultured mammalian cells from their substrate (cell detaching factor, CDF) and cytotoxicity.

Prominent pathogenic factors were the capacity of trichomonads interact specifically with mucin via a lectin-like adhesin[30] and then contact vaginal epithelial cells at which iron regulated surface proteins of *T. vaginalis*, adhesion protein (AP)[31], and lipophosphoglycan (LPG)[28]. Penetration of the epithelium by *T. vaginalis* also induces a specific interaction with extracellular matrix basement membrane glycoprotein.

The virulence of trichomonads is increased by iron. Iron modulates multiple aspects of *T. vaginalis*, including metabolic activity, cytoadherence and resistance to complement lysis due to proteinase degradation of C3 on the trichomonal surface[32]. *T. vaginalis* also binds to erythrocytes which provide both lipid and iron for parasites. During menstruation, the number of parasite tends to decrease. It has been suggested that the upregulation of adhesin level produced by the availability and additional iron may help the organism to persist through an unfavorable environment[33]. Otherwise, there is no strong evidence for the involvement of adhesins in pathogenicity. Adhesins alone are not sufficient to ensure adherence. Surface proteins also play a necessary role.

Surface proteins, proteins secreted by *T. vaginalis* were extensively examined with respect to interaction with human vaginal epithelial cells (HVEC). The components of *T. vaginalis* secreted proteins were identified as metabolic enzymes, proteases, and α-actin, which induced the expression of host

components, including interleukin 8, COX-2, and fibronectin[34]. Cysteine proteinases (CP) seen to be necessary for efficient adhesion protein mediated adhesion of parasites to targets. In addition, the study of Sommer in USA (2005) suggested that, CP-induced programmed apoptosis in human vaginal epithelial cells (HVEC) may be involved in the pathogenesis of *T. vaginalis* infection in vivo, may have important implications for therapeutic intervention[35]. Neutrophils are the predominant inflammatory cells found in the vaginal discharge of patients with *T. vaginalis* infection. However, one more factor can help *T. vaginalis* to survive and cause disease was the ability of inducing apoptosis of human neutrophils[36,37]. Otherwise, *T. vaginalis* induce TNF-α production in macrophages through nitric oxide (NO)-dependent activation of nuclear factor NF-κB, which involves in inflammatory process[38].

There are reports of other parasite products, described as cell-detaching factors, that are released by the parasite[39]. Although these products have not been extensively analysed, it is known that some of them do have trypsin-like activity. These factors are active on human cells, causing them to detach and round up. *T. vaginalis* released cell detaching factors and proteinases, and these parasite products degrade proteins such as laminin, vitronectin, and other components of the extracellular matrix. These affect the release of host cells from tissue. In addition, the levels of secretory leukocyte proteases inhibitor in patients with *T. vaginalis* infections are significantly lower than those in uninfected patients [40, 41], suggesting a possible role for parasite proteases enhanced risk of HIV infection associated with this parasite [42].

Data from recent studies suggested that some molecules may be produced by *T. vaginalis* and then delivered to target cells, mediating cytotoxicity through damage of their plasma membrane. By using electron microscopy, pores in

erythrocyte membranes caused by one of these molecules have been detected[43], displaying perforin-like activity of *T. vaginalis*[44].

An additional membrane-attacking molecule has recently been detected in *T. vaginalis*. A lytic factor (LF) is released by *T. vaginalis* that can destroy nucleated cells and erythrocytes and specifically degrades phosphatidylcholine, suggesting that it is a phospholipase A2[45].

The initial report of double-stranded RNA (dsRNA) viruses found in *T. vaginalis*[46]. Its presence correlates with variation of the expression of certain surface antigens, and loss of the dsRNA accompanied loss of antigen expression[47]. Recent reports confirm the presence of dsRNA virus in clinical isolates of *T. vaginalis*[48], with virus prevalence being as high as 82% in parasite isolates[49].

A recent study established the *T. vaginalis* α-enolase, TvBspA, TvPmp as a new surface-associated virulence factor and some of secreted molecules and peptidases can play the important role of *T. vaginalis* pathobiology [50,51].

In addition, we know that the natural vaginal epithelium and the bacteria flora are both profoundly affected hormonal status. After puberty, the vaginal stratified squamous epithelial cell are rich in glycogen and microbial flora is dominated by lactobacilli, causing a low pH (about 4.5). This condition is not suitable for the growth of *T. vaginalis*. In infected women, lactobacilli tend to disappear, and the pH increases about 6. This condition is good for *T. vaginalis* developing. Glycogen-rich squamous epithelial cells are also transiently present in born girls who are influenced by their mother's estrogen and it is these condition which are believed to allow colonization in neonatal trichomoniasis. As soon as the effect of exogenous estrogen decreased, glycogen disappears and the pH rise, these conditions persisting until puberty. The similar changes occur later after the menopause. That is not ideal for colonization by *T. vaginalis*.

In men, the hormonal influences were very little known, but asymptomatic infections were more common in men than women.

There were very little reports of trichomoniasis in prepubertal girls and post menopause women. The trichomoniasis cases in prepubertal girl were very rare and often in relation with sexual abuse, poor hygiene[52, 53]. For example, Street et al. in UK (1982) reported that, in his study using the whole cell antigen ELISA test and IgG antitrichomonal antibody positive was found in only three of the 99 children's sera examined[54]. Otherwise, Sharma et al. in USA (1997) shown the case of metronidazole - allergic postmenopausal woman was cured of vaginal trichomoniasis in association with discontinuation of estrogen replacement therapy. So hormonal manipulation should be studied proposed for the management of postmenopausal women with trichomoniasis who are allergic to metronidazole or who are infected with metronidazole-resistant strains of *Trichomonas vaginalis*[55].

In addition, *T. vaginalis* carring *Mycoplasma* may be linked to severity of mucosal damage, inflammatory symptoms, and consequences for reproductive outcome[56].

1.4. Immunology

Parasite's structure and pathogenicity have been extensively studied, but still little is known about the immunopathogenesis of trichomoniasis and the molecular mechanisms exploited by *T. vaginalis* to evade the immune system[57].

Clinical experience shows that re-infections by *T. vaginalis* can occur and in most cases the parasite was not rapidly cleared without treatment. A long-lasting sterile immunity clearly does not result, although the majority of women will develop modest levels of serum antibody. Antibodies to trichomonas are found in the serum of infected women, but the titers are in

general low. Ackers et al. (1975) considered their slow appearance to limit their value in the diagnosis of active disease [58].

Howere, using indirect fluorescent antibody test to detect antibodies to *Trichomonas vaginalis* in antenatal patients, Mason P.R. et al. (1979) reported that antitrichomonal IgA was found to be absent in the vaginal fluid of many patients with active *T. vaginalis* infections, while it was present in 42% of women with no evidence of existing trichomoniasis. Their results shown that IgG rather than IgM appeared to be the antibody class involved[59]. The study of Su K. S. in China (1982) also had the same results[60].

In addition, the study of Alderete J. P. et al. in USA (1984) proposed the use of enzyme linked immunosorbent assay (ELISA) for detecting antibody to antigenic *T. vaginalis* macromolecules using whole cells or an aqueous protein extract as antigen. Their results provided more knowledge of host immuno - response to *T. vaginalis* for future researches. It was suggested that, serum from experimental animals or infected people showed high concentrations of IgG, IgA, and IgM antibody to trichomonads. In women with acute trichomoniasis, only antibodies of the IgG and IgA class were detected in vaginal washes, no IgE antibody to trichomonads was found under a variety of conditions in serum samples from patients or even experimental animals[61]. Their results also shown that, 100% sera from patients, but none from sera of normal, uninfected women, possessed IgG to numerous trichomonad cysteine proteinases. This serum anti-proteinase antibody disappeared after pharmacological treatment and cure of patients[62]. In contrast, proteinases were detected in the vagina of some patients with trichomoniasis, and in most cases the proteinases were complexed with IgG. Patients without soluble proteinases in vaginal washes (VWs) also had antibody specifically to trichomonad proteinases.

Studies on immunogens have assessed the high degree of antigenic heterogeneity and phenotypic variability of the parasite. There were many more studies to understand immunological host response against *T. vaginalis*. In 1986 and 1991 the same objective study of Alderete J. P. shown that an immunoglobulin G type 2a (IgG2a) monoclonal antibody (MAb) produced complement-independent cytolysis of *Trichomonas vaginalis*. Synthetic peptides synthesized to this region demonstrated that the amino acid sequence DREGRD is important for antibody binding. Trichomonads that undergo phase variation during growth and multiplication may be capable of evading humoral immune mechanisms in their host[63,64]. Anti - *Trichomonas vaginalis* antibodies were investigated by Wos S.M. et al. in USA (1986) in patients with vaginal trichomoniasis to identify the predominant antibody isotype produced and to delineate clinically significant antigens. The total antibody content of serum samples from patients was determined by an enzyme-linked immunosorbent assay (ELISA) that employed anti-human immunoglobulin and isotype-specific antisera. The anti-*T. vaginalis* titer of all but two of these serum samples was greater than 200 (range, >200 to 12,800). By using an ELISA titer of at least 200 as a criterion, 21 of the serum samples contained antibodies of the immunoglobulin G (IgG) isotype, 17 contained IgM antibodies, and 6 contained IgA antibodies directed to the protozoan. These results add to the current understanding of the serological and secretory immune responses to *T. vaginalis*, as well as define potential antigens for use in immunodiagnostics[65]. The serologic screening of Addis M.F. et al. (1999) in Angolan women revealed that 41% of the women had IgG and IgM against the parasite. 94.4% of sera with anti-*T. vaginalis* IgG class antibodies were reactive against a common immunogenic protein of 115 kDa. The common immunogen was identified as the protozoan α-actinin[66].

Unfortunately, the immunity is not lasting, and it has been shown that *T. vaginalis* cysteine proteases present in the serum and vaginal secretions of symptomatic women can degrade IgG, IgM, and IgA[67]. In addition, the *T. vaginalis* cysteine proteases including CP30, induce apoptosis in vaginal epithelial cells[35], and in multiple mucosal immune cell types [57]. These results suggest that the acidic cysteine proteinase of *T. vaginalis* may play a dual role for parasite survival in conferring escape from host humoral defense by degradation of immunoglobulins, and in supplying nutrients to parasites by degradation of hemoglobin[68].

In contrast, Yap et al. (1995) demonstrated the relationship between serum antibodies to *Trichomonas vaginalis* and invasive cervical cancer patients by western blot technique. Antibodies to *T. vaginalis* were detected in the sera of 41.3% of invasive cervical cancer patients compared with only 5.0% of female controls. All the sera reacted strongly with the immunogenic surface membrane proteins of *T. vaginalis* of molecular weights of about 92 and 115 kDa, with variable reactivity to other immunogenic proteins of *T. vaginalis*[69].

More recent studies have found that *T. vaginalis* is associated with asymptomatic infections in 50% – 75% of sexual male partners[70]. Consequently, many men are unaware that they are infected with the parasite.

Serologic study of Sutcliffe S. et al. (2006) using a recombinant *Trichomonas vaginalis* α-actinin evidence of a history of trichomonosis also shown a positive association with incident prostate cancer[71].

Even though litle is known about immunological response in men, there are humoral immune responses against trichomoniasis in male patients.

1.5. Transmission

Humans are the only natural host for *T. vaginalis*. Nowadays, we know that trichomoniasis is one of sexually transmitted diseases. Therefore, use of both male and female condoms reduce the risk of transmission.

T. vaginalis can occasionaly be transmitted without sexual contact. Because *T. vaginalis* can survive for longtime outside the body if kept moist, the possibility of transmission via toilet seats, shared sponges or towels, communal bathing or living under poor and overcrowded conditions had been raised. This kind of transmission could explain for trichomoniasis diseases in children and in postmenopausal women without sexual contact[72].

Neonatal infection with *T. vaginalis* is infrequently reported, but has been noted to cause urinary tract infections and vaginitis in infants as premature as 28 weeks' gestation[73]. Neonatal trichomoniasis in girls were supported by their mother's hormonal influence.

1.6. Clinical features

1.6.1. Women

Trichomoniasis in women usually occurs during the reproductive years. Infection before menarche or after menopause is generally rare, and symptoms are mild and transient. The incubation period ranges from 3 to 28 days[74].

In women trichomoniasis may present as anything from an asymptomatic infection to an acute inflammatory disease, with a profuse, malodorous discharge. The severity of the discharge may alter over time. If untreated, the infection may be spontaneously lost or may persist for many months or years. The discharge is classically described as frothy, but it is actually frothy in only about 10% of patients. The color of the discharge may vary[75].

T. vaginalis may be found in the vagina and the exterior cervix in over 95% of infections, but is only recovered from the endocervix in 13%. In women,

signs of erythema are often present. Cervical and vaginal biopsies reveal areas of surface necrosis, erosion of the epithelium and infiltration by polymorphs and macrophages. According to European guideline on the management of vaginal discharge (IUSTI/WHO, 2011), the symptoms of trichomoniasis include: 10–50% asymptomatic, vaginal discharge, vulvar itching / irritation, dysuria, rarely low abdominal discomfort, vulvar erythema, vaginitis, vaginal discharge in up to 70%, frothy and yellow in 10–30%, approximately 2% 'strawberry' cervix, 5–15% no abnormal signs[76]. These signs and symptoms are cyclic and worsen around the time of menses.

In chronic infection, the predominant symptoms are mild, with pruritus and dyspareunia, while the vaginal secretion may be very scanty and mixed with mucus. This form of the disease is particularly important from the epidemiological point of view because these individuals are the major source of transmission of the parasite. Up to 25 to 50% of infected women are asymptomatic and have a normal vaginal pH of 3.8 to 4.2 and a normal vaginal flora. Although there is a carrier form, 50% of these women will develop clinical symptoms during the subsequent 6 months.

Although vaginitis is the most common manifestation of *T. vaginalis* infection in women, Bartholin's gland is an occasional focus of infection. Other complications associated with trichomoniasis include adnexitis, pyosalpinx, endometritis, infertility, low birth weight, and cervical erosion. Trichomoniasis is also associated with increased HIV transmission.

1.6.2. Virgin girl

Female infants can get infected during birth (Smith LM 2001)[72] or in sexually abused children[77, 78]. *T. vaginalis* can be transmitted to neonates during passage through an infected birth canal (2 to 17%), but the infection is usually asymptomatic and self - limited[79].

Smith K. (2001) found 12.9% *Trichomonas vaginalis* infection in teenage, non-virgin African American girls, and most of them were asymptoms[80].

1.6.3. Postmenopausal women

There was very rare case of trichomoniasis in postmenopausal women reported. In this group of patients with symptoms of vaginitis, the prevalence of vaginal candidiasis and trichomoniasis were lower than in reproductive age women and other causes (e.g. estrogen deficiency, nonanaerobic bacterial infections, local irritants or allergenes, and dermatologic conditions) need to be considered[81].

1.6.4. Men

The urethra is the most common site of infection. *T. vaginalis* has been reported in 13% of patients with non-gonococcal urethritis[82], but the organism has also been recovered from epididymal aspirates. Prostatic involvement has been reported, but its frequency and significance are not clear and it might be related with prostate carcinogenesis[71].

T. vaginalis has been detected in 66–77% of the male partners of infected women, and of those men, about 70% were asymptomatic[70]. In men the infection, although usually self-limiting and often asymptomatic, is associated with urethritis, prostatitis, epididymitis, reduced sperm function, and infertility reviewed in Benchimol et al., 2008[8]. There were spontaneous resolution of trichomoniasis and prolonged asymptomatic carriage occur in men with trichomoniasis[83].

In both sexes, dissemination beyond the lower urogenital tract is extremely rare and is not regularly found even in severely immunocompromised patients. A special characteristic of *T. vaginalis* infection is that often recurrent, with no lasting immunity, suggesting the importance of innate immunity.

1.7. Diagnostic techniques

Diagnosis of T. vaginalis's infection is being make on the basis of clinical symptoms, but in women, the characteristics of the vaginal discharge, including color and odor, are poor predictors of *T. vaginalis*[84, 85]. Detection of *T. vaginalis* organism is essential to the diagnosis of *T. vaginalis* because the clinical signs and symptoms are unreliable, and as many as 50% of cases are asymptomatic[86].

1.7.1. Microscopic examination

After a fresh specimen from the vaginal secretion is taken and transferred by a sterile wire loop to a glass slide, a cover slip will be put on it and then be examined using oil immersion high power, dark-field, or phase contrast microscopy. Microscopic examination has been a traditional technique to diagnose trichomoniasis with observation of motile protozoa in vaginal or cervical secretions.

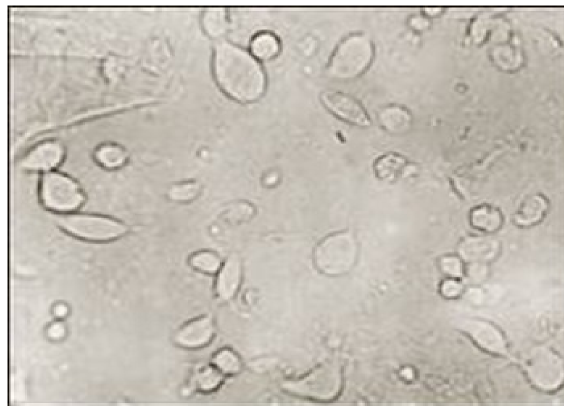


Figure 1.5. *T.vaginalis* in wet mount direct examination

Wet mount examination is straight forward and rapid, but more than 10^3/ml of live protozoa are required for detection[87]. Othewise the sensitivity of direct microscopy varies from as low as 38% to as high as 82%[88, 89] depend on the time, experience, the immediate examination of the specimen, and the loss of distinctive motility after the protozoan has been removed from body temperature.

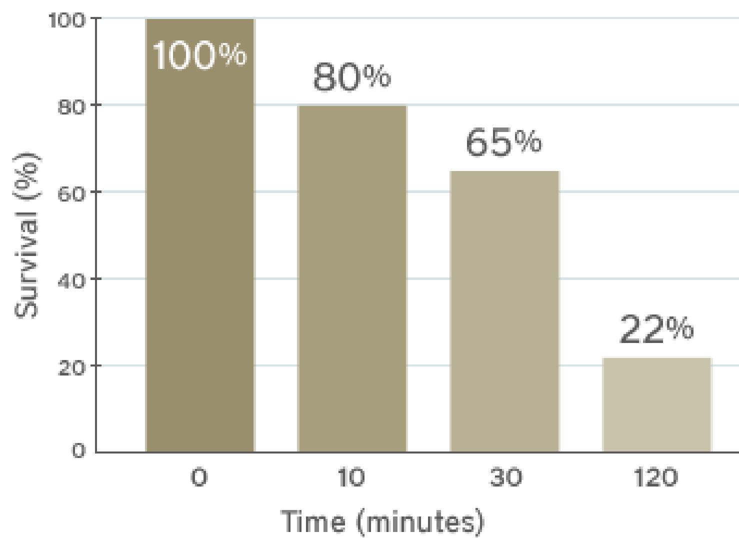


Chart 1.1. Decreasing shelf-life of *Trichomonas vaginalis*
on wet mount microscopic examination.[90]

The study of Patil M.J. (2012) in India showed the sensitivity and specificity of wet mount, which were 60% and 100%, respectively, whereas sensitivity and specificity of the In Pouch TV culture system were 73.33% and 100%, respectively when compared to PCR[91].

1.7.2. Cultural methods

The 'gold standard' for the diagnosis of trichomoniasis is broth culture technique using Diamond's medium. The minimum inoculum size required for a positive result is about 10 to 2 organisms/ml and the growth of the organism is easy to interpret[92]. However, there are inherent imitations to culture diagnosis[93]. An incubation period of 2 to 7 days is usually necessary to identify *T. vaginalis* in cultures, however during which time infected patients may continue to transmit the infection[94], and no culture systems are widely available to clinicians. To improve the acceptability of diagnosis by culture, a plastic envelope method was developed, which permits both immediate examination and culture in one self – contained system. The results are comparable to those of wet mount and culture techniques. Similar to the

plastic envelope method, the InPouch system is a two-chambered bag that allows the performance of an immediate wet-mount by microscopic examination through the bag, as well as a culture[95]. Levi et al. (1997) and Sood et al (2007) showed that the InPouch system is at least as sensitive as Diamond's modified medium for the detection of *T. vaginalis*[96, 97]. Borchardt et al. (1997) showed that this system is significantly more sensitive than either Diamond's modified medium or Trichosel medium[98].

The cell culture technique uses a variety of cell lines to recover *T. vaginalis* from clinical specimens[99]. Garber et al. (1987) used McCoy cells for the cultivation of *T.vaginalis* from clinical specimens and showed this method to be superior to the broth culture and wet-mount preparation since it was able to detect *T. vaginalis* at a concentration as low as 3 organisms/ml[87]. However, cell culture is not routinely performed, because it is expensive and not convenient for rapid diagnosis.

The systematic review by Patel et al. (2000) showed that the Diamond, Hollander, and CPLM culture media seem to be the most accurate, with sensitivities over 95%, thus could be used as reference standards[100]. Among these, Diamond's medium produces the maximal *Trichomonas vaginalis* growth in vitro[101].

1.7.3. Stain technique

Because cultivation methods are relatively slow and wet mount preparation yielded low sensitivity, the staining of parasites in fixed and unfixed smears was introduced.

According to Greenwood J. R. et al. (1981), staining techniques such as acridine orange is almost as sensitive as microscopic examination when specimens can be examined immediately after sampling[102].

Lowe G.H. (1965) shown that, examination of a Leishman-stained film yielded the highest proportion of positive results but probably failed to detect

trichomonads when their quantity was low. The best combination of methods was found to be Leishman film and culture: the positive yield was 99 %[103]. Papanicolaou staining holds considerable appeal in the diagnosis of trichomoniasis because it is routinely used in gynecologic screening for cytologic abnormalities, particularly in populations with a high prevalence of STD[104].

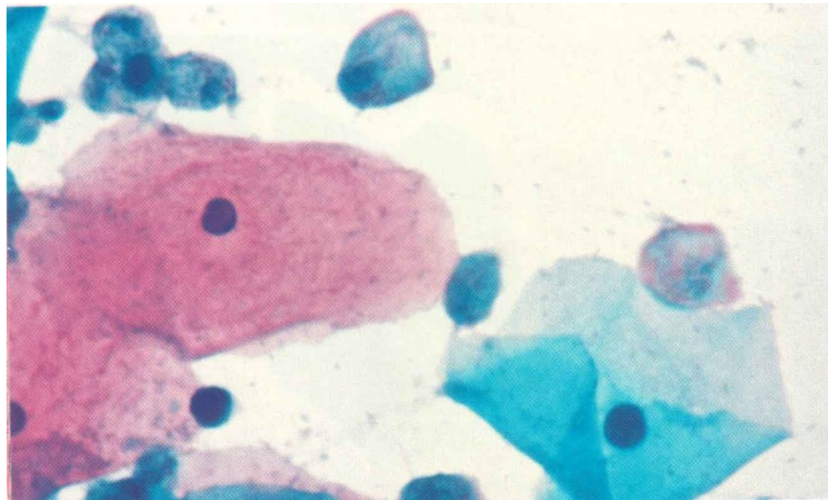


Figure 1.6. *Trichomonas vaginalis* in Pap smear[105]

However, the study of Karaman et al. (2008) in Turkey concluded that parasitological methods are more sensitive than Papanicolaou (Pap) staining methods in the diagnosis of *T. vaginalis*[106]. Perl G. (1972) in USA also reported a 48.4% error in diagnosis due to false-positive and false-negative findings when Pap smears were used as the sole criterion for diagnosis and treatment of *T. vaginalis* infection. Staining techniques have their limitations since *T. vaginalis* does not always appear in its typical pear-shaped form with flagella. It often appears as rounded forms resembling polymorphonuclear leukocytes, and occasionally the typical morphologic characteristics are lost during fixation and staining, making the etiologic identification difficult [107].

For diagnosis of *T. vaginalis*, El Sayed et al. in Egypt (2010) revealed that, wet mount showed reasonable sensitivity of 75.8%, acridine orange's sensitivity was 93.9% and specificity was 97.5%[108].

The limited power of culture and microscopic methods for the detection of *T. vaginalis* prompted the advance of the more sophisticated methods which can detect antigen, antibody, or nucleic acids in urethral or vaginal secretion.

1.7.4. Antibody-based techniques

Various techniques including agglutination, complement fixation, indirect hemagglutination, gel diffusion, fluorescent antibody, and enzyme-linked immunosorbentassay have been used to demonstrate the presence of antitrichomonal antibodies[,109].

Mathews et al. (1985) evaluated the indirect hemagglutination test and the gel diffusion test for efficacy in detecting antibodies in serum samples drawn from two population groups. Sera from patients attending a vaginitis clinic had a seropositivity rate of 69% by indirect hemagglutination and 34% by gel diffusion. Seropositivity rates among culture-positive patients were 78% with indirect hemagglutination and 43% with gel diffusion. A group of normal female hospital employees showed seropositivity rates of 30% by indirect hemagglutination and 3% by gel diffusion. Absorption of reactive sera with Trichomonas antigens reduced or abolished the serological reactivity, confirming the specificity of the test. Thus serological methods can provide a rapid, sensitive, and economical tool to study the epidemiology of this common protozoan infection[110].

Mason P.S. et al. (2001) reported that, enzyme immunoassay (EIA) was used to detect antibodies to *Trichomonas vaginalis* in sera from Zimbabwe. The EIA showed a sensitivity of 94 to 95% when compared with vaginal swab culture. The specificity was 77 to 85% in the two groups. The EIA may be useful for community surveys of trichomoniasis. Because *T. vaginalis* is a common sexually transmitted disease, the test may indicate behavior that increases the risk of STD transmission[111].

Any studies revealed antibody test were useful for diagnose *T.vaginalis* especially in asymptomatic patients and in general population, as well as in high-risks of STDs groups.

There were some specific antibodies against specific *T. vaginalis* antigens of including:

- 29 antigenic trichomonad polypeptides, with apparent molecular sizes ranging from 14 to >100 kilodaltons and with individual serum samples possessing different patterns of reactivity[65].
- The 115-kDa protein of the protozoan α-actinin (Addis M.F. et al 1999) [66].
- Surface protein (TV44) reactive with an IgA mAb (Mondodi et al. 2006)[112]
- Surface protein immunogen with a relative molecular mass of 230,000 daltons (230-kDa) (P230)[64,113].
- Parasite surface glycol-conjugate lipophosphoglycan (LPG) with distinct functions in the host immunoinflammatory response[28].
- The immunoreactive proteins included adhesion protein P65-1, α-actinin, kinesin-associated protein, teneurin, and 2 independent hypothetical proteins[114].

1.7.5. Molecular techniques

Recombinant DNA techniques have been increasingly used in clinical laboratories to improve the specificity and sensitivity of *T. vaginalis* diagnosis**.**

Many studies reported that, PCR appears to be the method of choice for the detection of genital *T. vaginalis* infections with high sensitivities and excellent specificities for both vaginal samples and male urethral samples[115, 116]. The primers for diagnose *T.vaginalis* were shown on table 1.1.

Table 1.1. The primers for diagnosis of *T. vaginalis*

Primer	Primer structure (5' – 3')	Segment
TVK3 /TVK7[117]	5′AT TGT CGA ACA TTG GTC TTA CCC TC3′ 5′ TCT GTG CCG TCT TCA AGT ATG C3′	312bp
TRICHO-F TRICHO-R[118]	5'CGGTAGGTGAACCTGCCGTT3' 5'TGCTTCAGTTCAGCGGGTCT3'	367bp
BTUB9/BTUB2[119]	5' CAT TGA TAA CGA AGC TCT TTA CGAT3' 5' GCA TGT TGT GCC GGACAT AAC CAT 3'	112bp
TvA5/TvA6[120]	5'GATCATGTTCTATCTTTTCA3' 5'GATCACCACCTTAGTTTACA3'	102bp
Tv1/Tv2[121, 122]	5' TAATGG CAG AAT CTT TGG AG 3' 5' GAA CTT TAA CCG AAG GAC TTC 3'	312bp
Tv-E650[123]	5´ GAGTTAGGGTATAATGTTGATGTG 3´ 5´ AGAATGTGATGACGAAATGGG 3´	650bp

However, repetitive sequences or amplification of the β-tubulin gene fails to detect some strains due to strain variation (Madico G 1998)[119].

Ertabaklar et al. (2011) revealed that, the wet mount had 60% sensitivity and 100% specificity, while PCR with primers targeting Tv-E650 showed 80% sensitivity and 97.95% specificity when compared with the culture method, regarded as the "gold standard"[124].

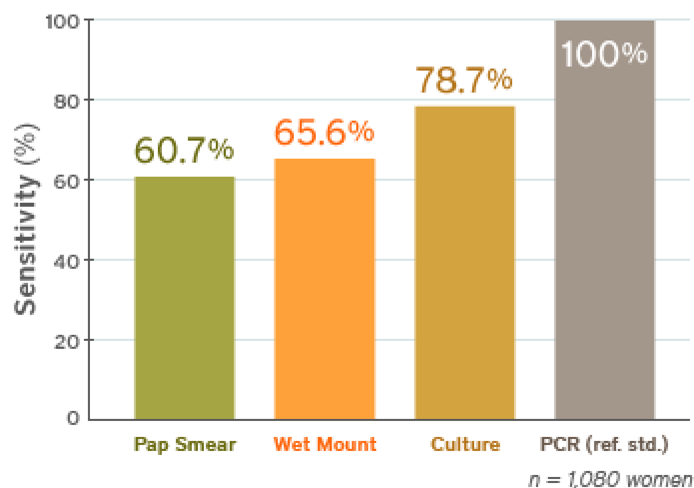


Chart 1.2. Sensitivity of microscopic examination to compare with culture, Pap smear and PCR[125]

In addition, Multiplex PCR appeared more useful for simultaneously detect coinfecting pathogens[126]. Multiplex PCR to detect both of *T. vaginalis* using primer TvA5, TvA6, *Mycoplasma hominis* using primer RNAH1, RNAH2 were high specific[127,128,129]. More researches are needed to evaluate the usefulness of these techniques for identifying asymptomatic carriers**.**

1.8. Treatment

The nitroimidazoles were the class of drugs useful for the oral or parenteral therapy of trichomoniasis. Metronidazole is available in the Vietnam for the treatment of trichomoniasis with few side effects and it is relatively inexpensive[130].

Recommended regimens

- Metronidazole 2 g orally in a single dose

OR

- Tinidazole 2 g orally in a single dose

Alternative regimen

- Metronidazole 500 mg orally twice a day for 7 days

In randomized clinical trials, the recommended metronidazole regimens have resulted in cure rates of approximately 90% -95%, and the recommended tinidazole regimens have resulted in cure rates of approximately 86% -100%. The treatment of sex partners might increase these reported rates. Randomized controlled trials comparing single 2 g doses of metronidazole and tinidazole suggest that tinidazole is equivalent to, or superior to, metronidazole in achieving parasitologic cure and resolution of symptoms. Treatment of patients and sex partners results in relief of symptoms, microbiologic cure, and reduction of transmission.

Metronidazole gel is considerably less efficacious for the treatment of trichomoniasis (<50%) than oral preparations of metronidazole. Topically applied antimicrobials (e.g. metronidazole gel) can not achieve therapeutic

levels in the urethra or perivaginal glands; therefore, use of the gel is not recommended. Several other topically applied antimicrobials occasionally have been used for treatment of trichomoniasis; however, these preparations probably do not have greater efficacy than metronidazole gel. Patients should be counseled for abstinence until they and their sex partners are cured[131, 132].

1.9. Therapy in resistant cases

When the metronidazole was first introduced in 1959, trichomoniasis cure rates approximated 95%, but within 2 years of its introduction, the first case of metronidazole resistance was reported in Canada and nitroimidazole resistance has now been observed in most areas of the world [133,134].

If single dose metronidazole 2 g failed and reinfection is excluded, the patient can be treated with metronidazole 500 mg orally twice daily for 7 days or single dose tinidazole 2 g. For patients failing either of these regimens, treatment with tinidazole or metronidazole at 2 g orally for 5 days should be considered (CDC 2006)[131].

Tinidazole yielded better efficacy against *T. vaginalis* isolates in vitro and has fewer side effects than metronidazole. However, because of the similarities in chemical structure, infections that are highly resistant to metronidazole may also fail to response after tinidazole therapy.

Resistant organisms are cosmopolitan in distribution and are of considerable concern as *Trichomonas* infections are linked to vaginal HIV transmission[135].

Alternative treatments for trichomoniasis resistance or allergy utilize compounds that are not absorbed well from the intestinal tract (paromomycin sulfate, furazolidone) or are not ingestible (povidone iodine) and therefore must be administered intravaginally. Despite these compounds are very effective against trichomonads in vitro, intravaginal therapy tends to be less efficacious than systemic treatment in contacting and killing all parasites.

Furazolidone, a nitrofuran, has also shown trichomonicidal activity in vitro[136]. Paromomycin has been used as a topical treatment for some patients with allergy to metronidazole or infections caused by metronidazole - resistant strains of *T. vaginalis*[137,138]. The other alternative drugs treatments for trichomoniasis have been investigated but until now it seem to be not effective or with high toxicity.

Since the introduction of 5-nitroimidazoles in the 1960s there have been reports of at least 100 metronidazole-resistant strains of *T. vaginalis* from the United States. Only under 20 metronidazole-resistant strains have been described from Europe. In addition, some preliminary reports have been published, for example, from Russia, and Africa[139], but no report so far in Vietnam.

Ryu et al. (1998) also demonstrated the existence of concordance between the genetic relationship and level of metronidazole susceptibility of *T. vaginalis* strains[140].

Some other studies shown that symbiosis of *Mycoplasma hominis* in *Trichomonas vaginalis* may induced in vitro metronidazole resistance[141].

In contrast, the study of Butler et al. (2010) in USA revealed that, *Mycoplasma hominis* infection of *Trichomonas vaginalis* is not associated with metronidazole-resistant trichomoniasis in clinical isolates[142].

2. RESEARCH OBJECTIVES

Trichomoniasis is a sexually transmitted disease caused by the parasitic protozoan *Trichomonas vaginalis*. The disease has a broad range of symptoms ranging from a state of severe inflammation and irritation with a frothy malodorous discharge to a relatively asymptomatic carrier state. It is the most common nonviral curable sexually transmitted disease, with estimated that the number of people suffering from curable STIs in the world per year is approximately 340 million[143], in which trichomoniasis was estimated 170 million people[144]. Trichomoniasis has been implicated in causing adverse pregnancy outcomes[145,146] and has been associated with an increased risk of human immunodeficiency virus (HIV) transmission[147,148]. Despite being a readily diagnosed and treatable sexually transmitted disease (STD), trichomoniasis is not a reportable infection, and control of the infection has received relatively little emphasis from public health STD control programs, such as in Vietnam. There are also no many studies in this field in Vietnam.

The introduction of metronidazole and others 5-nitroimidazoles available and effective, treatment of infections became possible. However, rare resistant metronidazole cases were reported[149]. The major problem in control of the disease depends on the accuracy of diagnosis. At present diagnosis is based on the microscopic demonstration of the parasite in wet smears, stained smears, culture media. The effectiveness of these methods is variable and depends on both the type of specimen taken and the processing of the specimen in the laboratory[150].

Trichomonas vaginalis induces humoral, secretory, and cellular immune responses in infected individuals[5]. Serological techniques therefore seem to be advantageous for diagnosing infections[59]. Many serological techniques (for

example, haemagglutination, complement-fixation, immunofluorescence, and radioimmunoassay) have been used to detect antibody to *Trichomonas vaginalis*. An enzyme-linked immunosorbent assay (ELISA) for detecting antibody to antigenic *Trichomonas vaginalis* macromolecules has been identified using whole cells of *T.vaginalis* had good sensitivity[61]. Otherwise, the efforts to getting more understand the pathogenesis and immunogen of trichomoniasis disease for prevention and control especially vaccination are emphasized. Nowadays, research into the development of a vaccine for *T. vaginalis* has shown some promise, elucidating a number of mechanisms by which protection could potentially be achieved. However, there are not much data about antibody against *T. vaginalis* in prospective studies.

Therefore, our research has been carried out with following objectives:

1. To estimate the prevalence of *T. vaginalis* in symptomatic and asymptomatic women of Hue City, Vietnam by clinical, wet mount microscopic, and serological examination.
2. To evaluate the antibody response against *T. vaginalis* during follow-up visits and determine the kinetic of antibody disappareance in sera of pharmacologically treated patients.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Study sites

This was a cross-sectional and prospective cohort study and was conducted from September 2010 to June 2012, at following settings:

- Gynecological Clinic of Hue University Hospital (GCHUH)
- Reproductive Healthcare Centre of Hue City (RHC HC)
- Parasitology Department, Hue University Hospital
- Carlo Urbani Centre, Department of Microbiology, Hue University of Medicine and Pharmacy
- Division of Experimental and Clinical Microbiology, Department of Biomedical Sciences – University of Sassari.

3.1.2. Study population

All indiviuals were provided information and signed informed consent on study procedure.

Symptomatic women

Women attending the Gynecological Clinic of Hue University Hospital, Reproductive Healthcare Centre of Hue City presenting symptoms of vaginitis and, discharge on clinical examination. After clinical examination, vaginal samples were collected for microbiologic investigations. The vaginal swab were immediately examined on microscope to confirm negative *T. vaginalis* infection. Since individuals in this group had symptoms suggesting vaginal infection, they are regarded as symptomatic women group. Number of patients enrolled in this group was 249 women.

Asymptomatic women

There were 534 asymptomatic women without vaginitis symptoms in community of Hue Province (Phu Vang district, Hue city, and mountainous

Nam Dong district) participated our study. They were introduced by volonteers of The Family Planning Program. The randomization of population was estabished in Vinh Thanh, Vinh Hien, Phu Mau, Phu Dien communes of Phu Vang District; An Hoa, An Dong, An Cuu, Phuong Duc communes of Hue City; Khe Tre, Huong Huu, Huong Loc communes of Nam Dong District. They were regarded as asymptomatic women since all of them were asymptomatic.

Trichomoniasis patients

All individuals were diagnosed vaginal trichomoniasis by microscopic examination. They were recruited from symptomatic and asymptomatic women in Gynecological Clinic of Hue University Hospital, Reproductive Healthcare Centre of Hue City, and in population of Hue City.

In total, 52 trichomoniasis patients were diagnosed by gynecological examination and wet mount microscopic examination, of these 46 patients agreed to participate into the study of evaluating the immuno-response against *T. vaginalis* during the follow up visits. Their blood and vaginal discharge samples were collected before pharmacological treatment. Sera were collected for detecting anti-*T. vaginalis* specific antibody by ELISA assay. The vaginal discharge samples were collected for *T. vaginalis* culture and multiplex PCR assay for *T. vaginalis, M. hominis* and *U. urealyticum.* They were also given the appointments for the follow up period of 5 months by 1-month interval. Every time, patients were clinically examined. The blood samples were again collected for monitoring the titers of anti - *T.vaginalis* specific antibody by ELISA assay and Western blotting analysis. The vaginal discharge samples were collected for evaluation of *T.vaginalis* presence or eradication by direct examination, culture and multiplex PCR.

Men

8 men who were the male partners of the trichomoniasis women were enrolled in this tudy.

Healthy men were the men who visited the Parasitology Department for examination of dermatophytose and students of Hue University of Medicine and Pharmacy. This group included 38 individuals.

Children

The sera of male/female children aged 2-10 years old without blood transmitted diseases were choosen from Biochemistry Depatment of Hue Central Hospital which originated from the patients being hospitalised at the Department of Pediatrics.

3.2. Methods

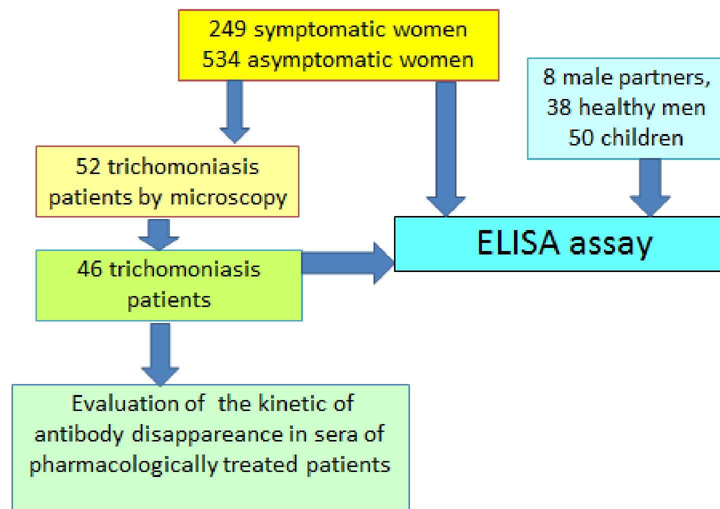


Figure 3.1. Scheme of study design

All individuals of study population were enrolled into the seroepidemiological study. Their sera sample were tested the IgG antibody to *T.vaginalis* by ELISA assay using the whole cell of *T.vaginalis* as antigen. Children' sera were used as negative control for ELISA assay.

Symptomatic and asymptomatic women were surveyed socio-demographic data, gynecological examination and followed up the trichomoniasis patients.

3.2.1. Socio-demographic data collection

Data on demography (age, occupation, level of education), risk sexual behaviour (number of sexual partners ever, condom usage) and the clinical signs and symptoms related to infection (recorded by the gynecologists) were collected by a questionnaire.

Levels of education included high level of education and low level of education. High level of education was defined as high school and college education or higher. Low level of education was defined as illiteracy, primary, or secondary education.

Sexual behavior

Depending on marital status and sexual behavior, we divided two different group of sexual behaviour including unsafe sexual behavior women, and safe sexual behavior women. Unsafe sexual behavior women were defined that herself or her partner had at least 2 sexual partners in last one year, and regarded as high risk. Safe sexual behavior women were defined that herself and her partner had no other partner in last one year, regarded as low risk individuals.

3.2.2. Gynecological examination

The clinical symptoms were noted including serious vaginal discharge, malodor vaginal discharge, yellowish-green frothy discharge, white discharge, purulent discharge, lower abdominal pain, vulvar itching, dysuria, dyspareunia, vulvar erythema, vaginal erythema, cervicitis, strawberry cervix. Asymptomatic patients were detected by gynecological examination before abortion, uterine IUD insertion or just by routine healthcare checkup.

The vaginal discharge were collected in two separate sterile tube with cotton stick, one for microscopy examination and culture *T. vaginalis* in Diamond

medium, the other for DNA extraction for multiplex PCR assay to diagnose *T. vaginalis, M. hominis, U. urealyticum.*

3.2.3. Treatment and follow-up

Trichomoniasis patients on the basis of wet mount direct examination on microscopy were provided free treatment, health education about STDs and subjected to follow-ups for examining titers of anti - *T. vaginalis* specific antibody by ELISA assay and Western blotting analysis.

Treatment regimens for trichomoniasis women and her partner were prescribed according to US Center for Disease Control's Sexually Transmitted Diseases treatment guidelines 2010, with metronidazole administered orally 500mg twice a day for 7 day. Alternative regimens were Tinidazole 2g orally in a single dose or Tinidazole 2g orally once daily for 2 days or Tinidazole 1g orally once daily for 5 days.

Recovered and unrecovered/re-infectious patients

Recovered patients were defined by the improvement of clinical symptoms and the eradication of *T.vaginalis* by microscopic examination, culture and PCR assay, confirmed during the course of 5 months of follow-up.

Unrecovered or re-infectious patients were defined by the persistence of clinical symptoms and/or *T. vaginalis* by microscopic examination, culture and PCR assay at any time during the course of 5 months of follow-up.

3.2.4. Laboratory examination

3.2.4.1. Microscopic examination

Vaginal and urethral discharge collected in sterile tube with cotton swab and saline solution 0.9% inside were examined as soon as possible after collecting for the presence of *T.vaginalis*.

Vaginal and urethral discharge mixed with saline solution 0.9% were smeared on the slide and examined on light microscope with the x10 and x40 objectives for motile flagellates.

In wet mount, flagellates can be identified by their pattern of movement. Trichomonads trophozoites was confirmed by ovoid-shaped parasites which slightly larger than polymorphonuclear lymphocytes (PMNs), moving with nervous, jerky or jumpy movement and undulating membrane.

On microscopy examination, we also notified the polymorphonuclaires, "clue cells", and *Candida.*

The presence of "clue cells" more than 20% of epithelial cells meaning "clue cells" positive was taken as evidence of bacterial vaginosis (BV)[151].

The diagnosis of candidasis was based on the presence of blastoconidia or pseudohyphae and neutrophils. PMNs increasing in vaginal secretion was defined by in a ratio of PMNs to squamous epithelial cells more than 1:1.[152]

3.2.4.2. *Trichomonas vaginalis* culture in Diamond medium

Vaginal and urethral discharge collected by sterile cotton swab was immediately cultivated on Diamond's medium (Diamond, 1957) (pH 6.6) with 1000UI/ml Penicilium, 100µg/ml streptomycin, 250µg/ml fluconazole and 10% fetal bovin serum (Invitrogen, No. 1600-044) in falcol tube (Corning No.430791) or microplate 24 well (IWAKI No. 3820-024) at 37 ^{o} C in the CO_2 5% incubator in 7 days after microscopy. The cultures were examined microscopically on day 2, day 4, day 6, and day 7 after inoculation. A positive result was defined as the presence of motile trichomonads at any time, a negative result was defined as the absence of motile trichomonads at all readings.

3.2.4.3. Preparation of serum samples

1 ml nonheparinized blood was collected in sterilized vials. Serum was separated and stored at - 20°C until test for antibody.

3.2.4.4. Preparation of ELISA plates

The ELISA assay was carried out following a method described by Alderete P.J. (1984)[61], and Mason P. R. (2001) using the G3 strain of *T. vaginalis*[111].

This isolate originated from United Kingdom, and is characterized by being free from mycoplasma infection. Long-term cultures in T25 flask (Becton Dickinson England No 353014) using standard protocol were maintained using mycoplasma-free Diamond's medium. Parasites in logarithmic growth were harvested, washed three times in phosphate buffered saline (PBS) and suspended at 1-1.5 x106 cells/ml. Aliquots (50 µl) were added to the wells of microtitre plates. The plate was incubated overnight at room temperature (RT). The well were added 50µl /well of methanol, fix for 10minutes at RT. After removing methanol from the plate by aspiration, the well were washed 3 times by PBS-tween 20 0,05% (200µl/well). After adding 100µl/well of PBS – bovin serum albumin (BSA) (A 2153-Sigma Aldrich) 1%, the well were incubated for 2 hours at RT or overnight at 4^0C. Then the well were washed with distilled water. After drying in air about 1-2hours, the well were stored at 4^0C until use.

3.2.3.5. ELISA assay

All sera samples were collected were tested by ELISA assay using the whole cell of *T. vaginalis* as antigen for detecting anti – *T. vaginalis* IgG antibody.

Each ELISA plates had the positive, negative sera control and one white well with PBS alone. Positive sera control were obtained from trichomoniais patient, of whom had an active *T. vaginalis* infection, based on microscopy and culture, at the time of blood collection. Negative sera control were obtained from children sera or healthy women. Healthy women had clearly no history of vaginitis, and her vaginal sample was not found *T. vaginalis* based on microscopy and culture, at the time of blood collection.

Sera were diluted 1:100 in PBS solution, and 100µl were added to each well and the plate were incubated for 90 minutes at RT. Washing the well 3 time by 200µl/well of PBS with 5% of tween 20 (250µl tween 20 in 500ml PBS). Anti human IgG Fc specific alkaline phosphatase conjugate (No. A9544 of

Sigma Aldrich USA) were diluted 1:30,000 in PBS-BSA 1% immediately before adding 100µl into each well. After incubating for 60 min at RT, the well were washed 3 time by 200µl/well of PBS with 5% of tween 20. One tablet of ELISA substrate (p-Nitrophenyl phosphate, product No.N2765 of Sigma Aldrich USA) were diluted in 20ml AP buffer pH 9,5. After adding 100µl/well of substrate solution, the well were read the optical density (OD) at 405 nm in 15-30 minutes by ELISA reader of Biorad 680.

3.2.3.6. DNA extraction from vaginal swab using DNA extract kit (Viet A Company, Vietnam)

The vaginal cotton swab were transferred into Eppendorf tube containing 318µl TE pH 8,0 + 80µl SDS 10% + 2µl proteinase K (20mg/ml). After incubating at 65°C for 30minutes, the cotton swab were removed. The solution were added by 400µl phenol-chlorophorm-isoamilic alcohol (25:24:1) and mixed well. After centrifuged at 14000rpm for 10minutes, the solution were carefully removed the supernatant layer to a new tube, being careful to avoid the interface.

After adding 2 volume of cold absolute ethanol and 0,1 volume of 3M sodium acetate pH 5,2, the tube were placed at -70°C for at least 2 hours, or at -20°C overnight. After centrifuge at 14000 rpm for 15min in 4°C, the pellet DNA were washed with cold 70% ethanol. After discarding the supernatant carefully and letting tube be completely dried at room temperature (place eppendorf tube downward on the tissue paper). The DNA were dissolved in TE buffer (50µl) and keeped freezing at -20^{0} C if have not do PCR yet.

3.2.4.7. Multiplex PCR assay to diagnose *T. vaginalis, M. hominis, U. urealyticum*

Multiplex PCR was permorming following the protocol of Diaza N. et al 2010[129]. The optimized protocol for the Multiplex PCR consisted of a reaction mixture of 25 µM containing 20mmol/L Tris, pH 8.4, 50mmol/L KCl,

2.5mmol/L $MgCl_2$, 200 µM of each deoxyribonucleotide, 1.5 U of recombinant Taq polymerase (Invitrogen, Milan, Italy), 12.5 pmol of primers TvA5, TvA6, Uu, Uu2 and 2.5pmol of primers RNAH, RNAH2 10µM and 2µl of DNA.

Primers		Primer structure (5'-3')	Amplicon size
T.vaginalis	TVA5	GATCATGTTCTATCTTTTCA	102bp
	TVA6	GATCACCACCTTAGTTTACA	
Ureplasma urealyticum	Uu1	AGAAGACGTTTAGCTAGAGG	541bp
	Uu2	ACGACGTCCATAAGCAACT	
M. hominis	RNAH1	CAATGGCTAATGCCGGATACGC	334bp
	RNAH2	GGTACCGTCAGTCTGCAAT	

Amplifications were performed in a PCR thermal cycler with an initial denaturation step of 94^0C for 30 seconds, annealing at 55^0C for 30 seconds, extension for 1 min at 72^0C, and final extension step of 8 minutes at 72^0C. The multiplex PCR products were electrophoresed through a 1% agarose gel in TBE and visualized with an ultraviolet transilluminator after ethidium bromide staining.

Samples containing a 102-bp fragment were considered positive for *T. vaginalis*, 334bp fragment for *M. hominis*, 514bp fragment *for U. urealyticum*.

3.2.4.8. Western blot to find specific antibody against *T. vaginalis*

Total *T. vaginalis* SS-22 protein preparations were obtained by trichloroacetic acid precipitation[153], electrophoresed by SDS-PAGE, and transferred onto a nitrocellulose membrane. Vietnamese sera of following up trichomoniasis patients affected by trichomoniasis were diluted 1 : 200. As a control, the sera from Vietnamese women negative for *T. vaginalis* by both ELISA and wet-mount. After incubation, membranes were washed and incubated with

antihuman IgG or IgM rabbit immunoglobulins conjugated with alkaline phosphatase (Sigma, St. Louis). Bound antibodies were detected with chromogenic substrates[66].

3.3. Ethical issue

Study protocols were approved by Hue University of Medicine and Pharmacy Institutional Review and Ethical Board.

3.4. Data analysis

Statistical analysis was performed using Microsoft Excel 2010 and Medcalc software.

Comparison of proportions between two rate were calculated by Chi-square test.

Comparison of two mean were calculated by Independent sample T – test for evaluation ELISA assay.

Comparison of two mean of following up sera antibody titers were calculated by Kruskal – Wallis test because of the small number of patients.

ROC analysis were used to evaluate sensitivity and specificity of ELISA assay.

ROC analysis were also used to evaluate the relation between the following up sera antibody titers and clinical symptoms. Levene's test for equality of variances, ANOVA test for evaluation the relationship, Student-Newman-Keuls test for all pairwise comparisons.

All reported confidence intervals were two-sided 95% confidence intervals and P - values <0.05 were regarded as statistically significant.

4. RESULTS

4.1. Epidemiology of trichomoniasis

4.1.1. Demographic characteristics of population

The baseline sociodemographic characteristics and sexual history of each groups were shown in the table 4.1.

Table 4.1. Demographic characteristics of the study groups

	Symptomatic women n = 243	Asymptomatic women n = 534
Mean age	38±10 (20-60)	37±7 (20-49)
Geographic area		
Urban	52.3%	28.3%
Rural	47.7%	71.7%
Education level		
High level	39.1%	24.3%
Low level	61.9%	75.7%
Sexual behavior		
Safe sexual behavior	46.9%	90.8%
Unsafe sexual behavior	53.1%	9.2%

Since the number of men was limited, and most of them being student, we did not evaluate their demographic characteristics. The mean age, and education level of the two women groups were similar.

4.1.2. Prevalence of *T. vaginalis* infection

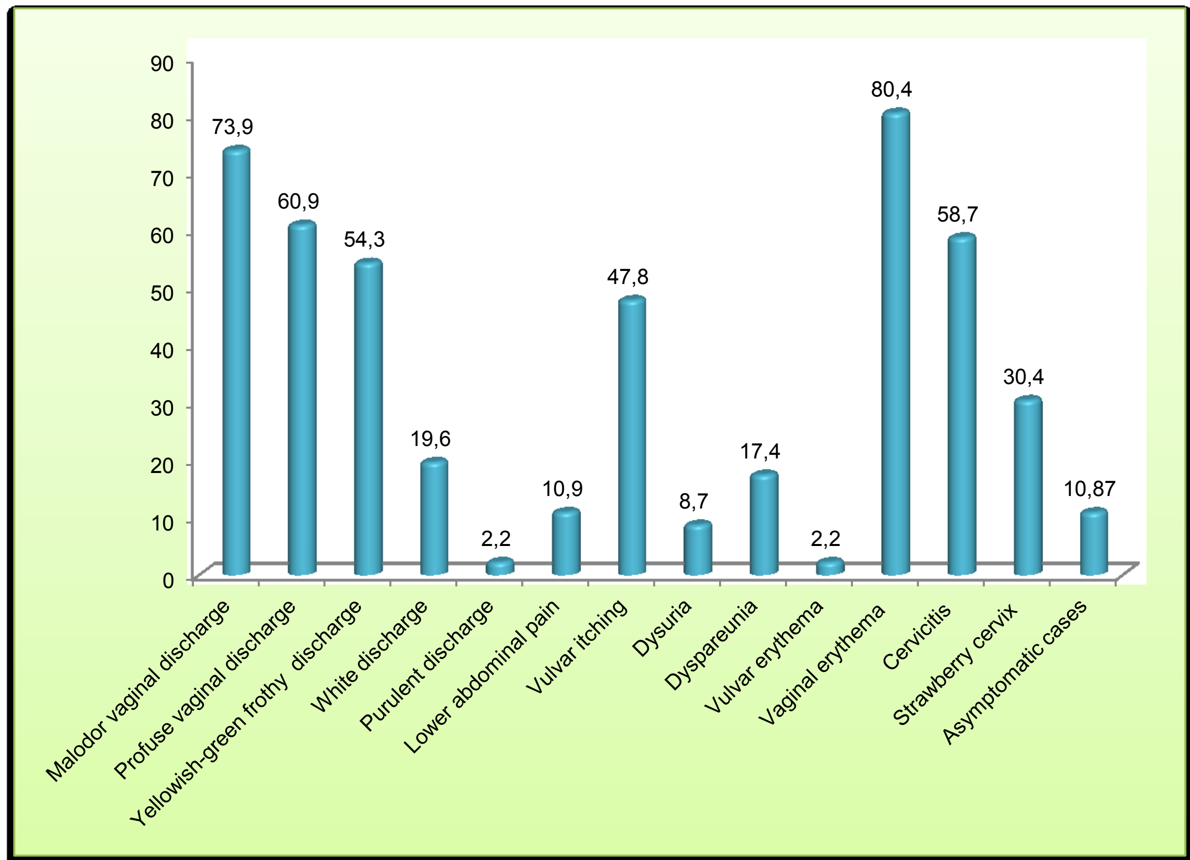
Table 4.2. Prevalence of *T. vaginalis* infection in subgroups

Subgroups	Number of case	Rate (%)	p	x^2
Symptom				
Symptomatic women	48/249	19.3	<0.0001	92.117
Asymptomatic women	4/534	0.7		
Geographic area				
Urban	30/283	10.6	0.0014	10.227
Rural	22/500	4.4		
Education level				
Low level	31/228	13.6	<0.0001	23.437
High level	21/555	3.8		
Sexual behavior				
Safe sexual behavior	19/603	3.2	<0.0001	48.368
Unsafe sexual behavior	33/180	18.3		

The prevalence of trichomoniasis diagnosed by microscopic examination in symptomatic and asymptomatic groups were 19.3% (42/243, 95% CI = 14.6% - 24.8%) and 0.7% (4/534, 95% CI = 0.18% - 1.8%), respectively. There were statistically significant differences in the distribution of prevalences among different geographic areas (urban vs. rural), educational levels (low vs. high level) and sexual behaviour (safe sex vs. unsafe sex).

4.1.3. Clinical features of trichomoniasis patients

Chart 4.1. Frequency of symptoms in trichomoniasis patients



From 46 trichomoniasis patients disgnosed by clinical and microscopic examination, 91.3% of patients were in reproductive age, with the mean age 37±9 (20-60). There were 4 (8.7%) menopausal patients.

The most prevalent symptoms were vaginal erythema (80.4%), malodorous vaginal discharge (73.9%), profuse vaginal discharge (60.9%); cervicitis (58.7%), and yellowish-green frothy discharge (54.3%), and 10.9% asymptomatic cases.

4.1.4. Co-infection

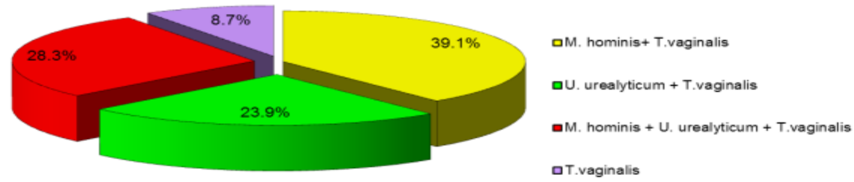


Chart 4.2. The co-infection of *T. vaginalis*, *M. hominis, U. urealyticum*

Co-infection with *M. hominis, U. urealyticum* and both of them has been recorded in 39.1%, 23.9% and 28.3%, respectively. There were only 8.7% infected *T. vaginalis* alone.

4.2. Seroepidemiology of trichomoniasis

4.2.1. Comparison of anti – *T. vaginalis* specific antibody reaction between trichomoniasis patients from different groups.

Table 4.3. Optical density (OD) among different groups

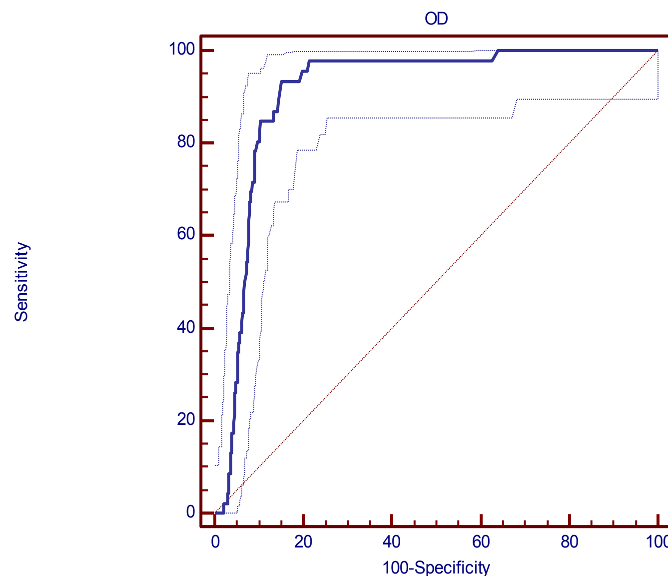
Group	n	OD (mean ±1SD)	P	*t*
Children	50	0,080 ± 0,01$_{(1)}$ (0.07-0.12)	$P_{1vs2} < 0.001$ $P_{1vs3} = 0.007$	8.23 2.803
Healthy men	38	0.122± 0.034$_{(2)}$ (0.072-0.20)	$P_{3vs2} = 0.03$ $P_{2vs4} = 0.002$	2.19 3.18
Male partners	8	0.094 ± 0.026$_{(3)}$ (0.068-0.175)	$P_{3vs4} = 0.0006$ $P_{3vs5} < 0.0001$	3.05 5.71
Symptomatic women	201	0,144 ± 0,04$_{(4)}$ (0.074-0.401)	$P_{4vs5} < 0.0001$ $P_{1vs4} < 0.0001$	12.245 11.2
Trichomoniasis women	46	0,238 ± 0,07$_{(5)}$ (0.117-0.475)	$P_{5vs1} < 0.0001$ $P_{2vs5} < 0.0001$	15.79 9.34

The mean OD of trichomoniasis women group was statistically significant higher than the mean OD of each other group with P < 0.0001. There were also statistically significant differences in mean of OD between each two groups.

Positive and negative control sera

Sera from trichomoniasis patients to set up ELISA test. The mean ±1SD of control positive sera were 0.306 ± 0.120 (0.175 - 0.582).

Negative sera control were obtained from healthy women, of whom had clearly no history of vaginitis, and her vaginal sample was not found *T. vaginalis* based on microscopy and culture, at the time of blood collection. The mean ±1SD of control negative sera were 0.123 ± 0.03 (0.087 – 0.173)



Graph 4.1. ROC curve of ELISA test using direct examination as the standard diagnosis of *T.vaginalis*.

Area under the ROC curve (AUC) was 0.912 (95% CI=0.890 - 0.931), P <0.0001.

Table 4.4. Sensitivity and specificity of ELISA test

at difference cut-off values

Cut-off value	Sensitivity%	Specificity%
>0.168	93.48	83.50
>0.17	93.48	84.33
>0.172	93.48	84.60
>0.173	93.48	84.74
>0.174*	**93.48**	**84.88**
>0.175	89.13	85.44
>0.176	86.96	85.71
>0.177	86.96	86.13

The best cut-off value of OD in our study is 0.174 with sensitivity of 93.48% and specificity of 84.88%.

With this cut-off value, there were no negative sera that gave a positive OD in all of tests, and no positive sera that gave a negative OD in all of tests.

These data demonstrate the reliability of ELISA test used for seroepidemiological studies.

4.2.2. Seroprevalence of *T. vaginalis* antibody in subgroups of study

Table 4.5. The rate of seropositivity in *T. vaginalis* in subgroups

Group		N	Rate %	P	X^2
Symptom					
	Symptom	76/243	31.3	<0.0001	34.096
	Asymptom	71/534	13.3		
Geographic area					
	Rural	73/499	14.6	0.0001	16.003
	Urban	74/278	26.6		
Education level					
	Low	97/552	17.6	0.1665	1.914
	High	50/225	22.2		
Sexual behavior					
	Safe sex	84/599	14.0	0.0019	9.688
	Unsafe sex	63/278	22.7		
Gender					
	Men	4/46	8.7	0.123	2.375
	Women	147/777	18.9		
	Children	0/50	0		

Seroprevalence in subgroups were statistically significant differences in the distribution among symptomatic vs asymptomatic women, different geographic areas (urban vs. rural), and sexual behaviour (safe sex vs. unsafe sex).

Only 3/38 (7.9%) sera from low risk men of trichomoniasis were seropositive compared with 1/8 (12.5%) from high risk men (with P = 0.8). In total, 147/777(18.9%) sera from women were seropositive compared with 4/46 (8.7%) from men (P = 0.123).

4.3. Follow-up of selected patients

4.3.1. Antibody response against *T. vaginalis* during follow-up visits

Before pharmacological treatment, there were 46 trichomoniasis patients. However, only 30 of them attended the follow-up visits. Because of several reasons, there were only 6 patients attended 5 follow-up visits during 5 months. The interval periods were show on the table 4.6.

In recovered patients, which are defined by improvement of clinical symptoms and the eradication of *T.vaginalis* by microscopic examination, culture and PCR assay, confirmed during the course of 5 months of follow-up, the symptom improved gradualy over time. The malodor, yellowish-green frothy discharge, serious vaginal discharge were noted that improved by 1 month. In the first follow-up visit after 1 month, there were 13/28 patients still having vaginal erythema and cervicitis by speculum examination and, at the second follow-up visit there were 6/17 patients having vaginal erythema and cervicitis symptoms by speculum examination. At the third visit, even though some cases were still having OD ratio greater than 1, 100% of clinical symptoms improved.

In unrecovered/reinfectious patients (described in Materials and Methods), the symptoms remained unchanged during the follow up visits.

OD ratio of IgG antibody against *T. vaginalis* of patients and OD of negative control (NC) was used as baseline to compare the titers of antibody IgG against *T. vaginalis* in follow-up patients.

Table 4.6. OD ratio of patients (Pa)/negative control (NC) during 5 - month follow-up

OD ratio of Pa/NC						
Patient number	Pretreat.	1 month	2 months	3 months	4 months	5 months
28	**0.95**	0.95	0.95			
66	**2.01**	**2.17**	**1.63**	1.6	0.95	1.13
68	**2.76**	**1.7**				
69	**1.89**	**1.98**	**2.83**	**1.94**	**3.59**	**1.27**
79	**2.64**	**4.57**	**3.35**	**6.95**	**2.55**	**2.94**
85	**2.9**	**4.28**		1.33	1.35	1.09
84	**1.4**	**1.4**	0.82	0.88	0.86	0.88
227	**1.91**	**1.01**	**1.04**	0.98	0.93	0.107
360	**2.16**	**1.92**	**1.87**		1.73	1.73
361	**1.27**			1.1		
362	**1.65**	**1.4**	**1.28**	**1.3**	**1.35**	**0.65**
363	**1.12**	**1.13**	**1.18**	1.19	0.84	
365	**1.1**	**1.13**		0.79		0.72
368	**1.08**	**1.16**		0.82		
370	**0.99**	**1.12**	0.72			
372	**1.8**	**1.51**	0.75	0.51		
374	**1.37**	0.88				
376	**0.92**	0.98	**1.34**	1.29		0.71
378	**1.03**	0.68				
379	**1.16**	0.97		0.83		
380	**1.1**	0.71				
383	**1.17**	0.66	0.57			0.65
384	**1.36**	**1.19**	**1.18**		0.62	0.58
386	**1.1**	0.95				
387	**1.22**	**1.21**			1.11	0.94
388	**1.13**	1.2				
389	**0.81**	0.86				
392	**1.16**			0.84	0.77	0.68
574	**0.98**	1.43	0.85		0.76	0.72
961	**0.93**	0.89	0.98			

Dark color: presence of clinical symptoms; light color: improvements of clinical symptoms

The clinical symptoms of patients included in follow up studies were also shown on the table 4.6. The dark color show clinical symptoms and the light color shows the improvement of clinical symptoms during the follow up periods.

In un-recovered/reinfectious patients, the antibody titer stay at high level during the follow-up. In recovered patients, the antibody titer decrease over time.

4.3.2. Host immunological response in different groups

Table 4.7. Comparision of the OD ratio during follow-up and between the recovery and unrecovery group (Kruskal – Wallis test).

	pretreated	1m	2ms	3ms	4ms	5ms
Recovered group	1.37±0.5	1.28±0.7	1.07±0.4	0.95±0.4	0.99±0.3	0.83±0.4
	n = 27	n = 25	n = 13	n = 12	n = 10	n = 12
P	0.06	0.03	0.03	0.03	0.02	0.04
t	3.6	4.6	4.9	5.3	5.2	4.0
Unrecovered group	2.06±0.5	2.65±1.7	2.49±1.1	3.40±3.1	2.50±1.1	2.11±1.2
	n = 3	n = 3	n = 3	n = 3	n = 3	n = 2

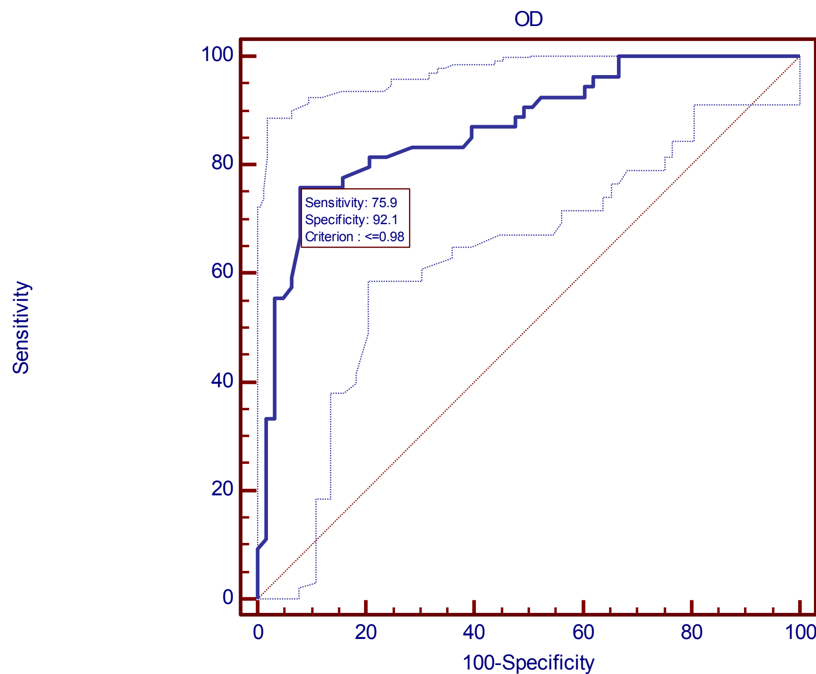
The mean of OD ratio of Pa/NC in recovered patients gradually decrease over time and became lower than 1 at the third month of follow–up. In contrast, the mean of OD ratio of Pa/NC in un-recovered/reinfectious patients go slightly down at the fifth month but maintains an high level.

A general decrease of the antibody response was observed after pharmacological treatment in patients that do not show symptoms or *T.vaginalis* infection. On the contrary, drug resistant (or re-infected) cases do

not show antibody titre decrease, confirming that presence of antigenic stimulus is mandatory to induce and maintain antibody response.

Related to the kinetics of antibody titre, data from this study also demonstrated that the "shelf life" of specific antibody response is 5-7 months.

4.3.3. Relation between antibody IgG against *T. vaginalis* titers with clinical symptoms



Graph 4.2. ROC illustration the relation between titers of IgG against T. vaginalis with clinical symtoms. Levene's test for equality of variances ($P < 0.001$), ANOVA test for evaluation the relationship ($P < 0.001$), Student-Newman-Keuls test for all pairwise comparisons.

Table 4. 8. Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	Specificity
<=0.94	59.26	93.65
<=0.95	66.67	92.06
<=0.97	68.52	92.06
<=0.98*	75.93	92.06
<=0.99	75.93	90.48
<=1.01	75.93	88.89
<=1.03	75.93	87.30

This data showed that the decreasing of antibodies titers (0.98) related to the healing of clinical symptoms (Se =75.93, Sp =92.06) .

4.3.4. Western blotting analysis specific antibody during follow-ups

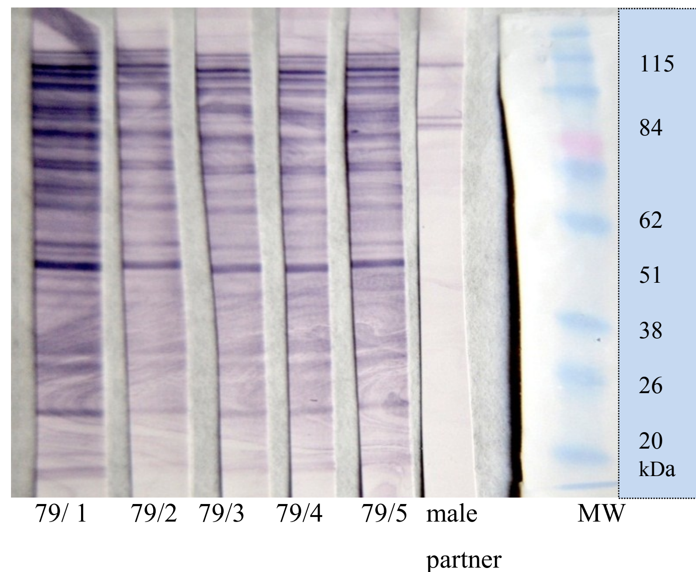


Figure 4.1. Immunoblot patterns of patient number 79 (case number 2: recurrent exposure) during the follow up periods and her husband. Representative sera with high IgG response obtained by probing a total *Trichomonas vaginalis* protein preparation. The number from 1 to 5 represent

months after treatment and the last band is serum samples from male sexual partner. Molecular weight (MW) markers are shown on the right.

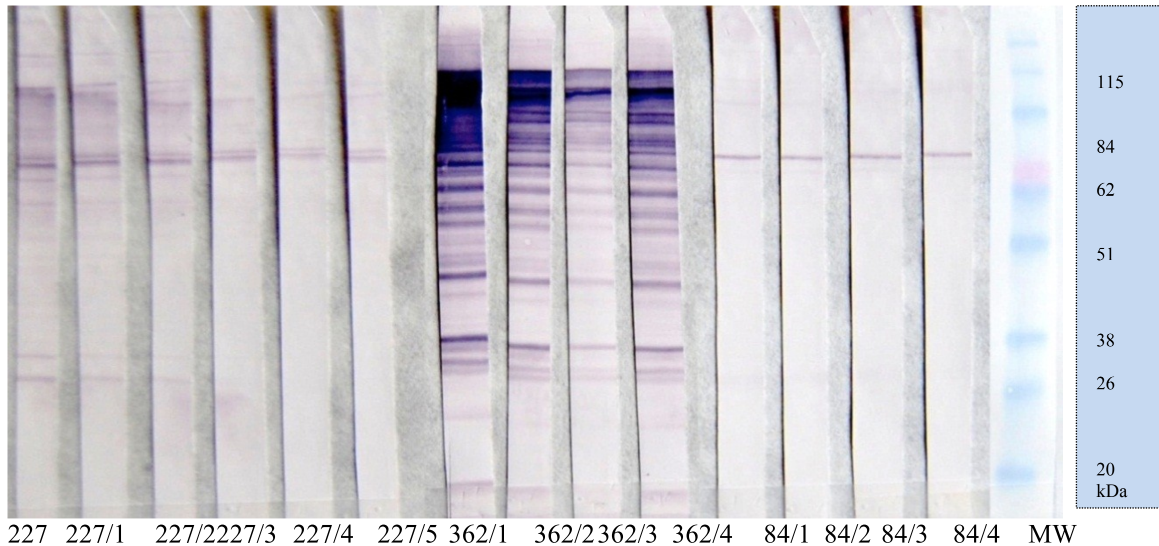
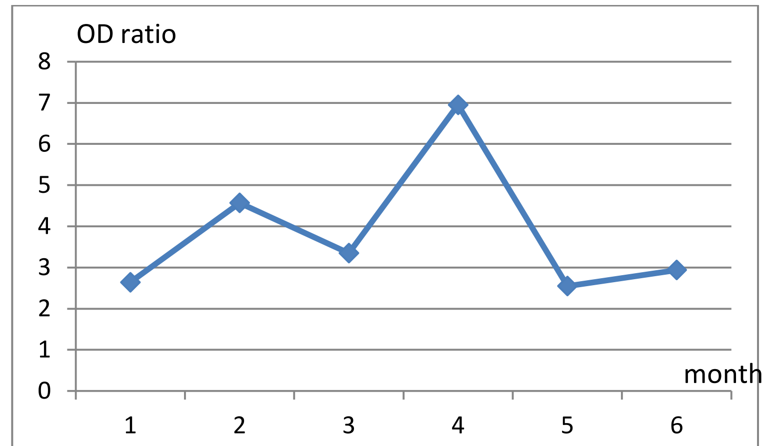


Figure 4.2. WB analysis of 2 recovering patients (No 227 and No 84: "natural cured") and re-infected patient (patient number 362) during follow-up periods. The number from 1 to 5 are months after treatment. Molecular weight (MW) markers are shown on the right.

There are the significant correlations between Western blotting, ELISA assay and clinical symptoms. The high OD ratio of Pa/NC of current trichomoniasis and unrecovery patients produced many more bands than the low OD ratio of Pa/NC cured patients at all MW ranges particularly evident in the high-molecular weigh range. The OD ratio of Pa/NC of cured patients decreases over time and is confirmed by the disappearing of antibody to *T.vaginalis* in WB analysing. However, antibody to *T. vaginalis* antigen between 84-115kDa are still present. The same results can be observed in the band pattern on WB analysing of male partners sera (Figure A.4).

4.3.5. Cases presentation

Case 1



Graph 4.3. OD ratio curve during the follow-up period of recurrent exposure (Patient number 79).

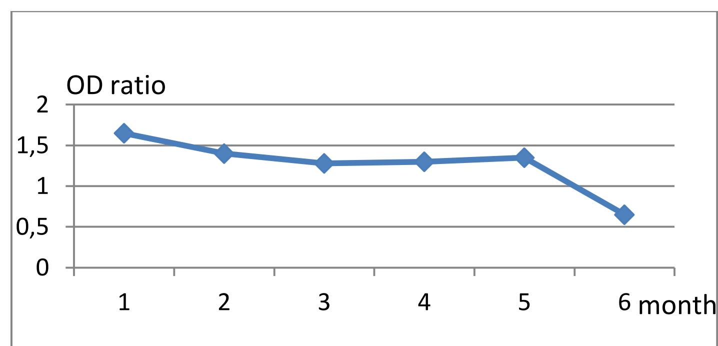
A 52 year - old, married women, still having menses. She had a diagnosis of trichomoniasis with yellowish-green frothy discharge, strawberry cervix, vaginal erythema. The vaginal pH was 6, and numerous PMNs and *T.vaginalis* were observed on DE. Mutiplex PCR shown *T.vaginalis* co - infectino with *M. hominis*.

Her husband had several sexual partners. No urethritis symptoms and *T. vaginalis* culture from urine was negative. ELISA testing of his sera was 0.142/0.139 (1.02).

The patient was given standard metronidazole treatment and during the 5 follow up visits she still had strawberry cervix, vaginal eryrthema, but microbiological and PCR testing for *T. vaginalis* were negative (Figure A.5). Other STI agents were not found and vaginal cytological examination was normal. During follow-up the patient, due to persistence of clinical findings,

was still using oral metronidazole 500mg b.i.d. for 7 days after ginecological examination. During the follow-up period she had sexual intercourses with her husband without using condom. Her husband did not completed treatment. At the final monitoring time, eventhough *T.vaginalis* was not found by any testing, her OD ratio Pa/NC was still higher than 1(2.94). Six months after ending follow-up, she came back with trichomoniasis and with OD Pa/NC ratio of 1.95. WB analysis for antibody to *T.vaginalis* shows similar pattern during all follow-up. Her husband serum reacted slightly with the total T.vaginalis antigen, with a more clear reaction with antigens from 84 to 115kDa (figure 4.1)

Case 2



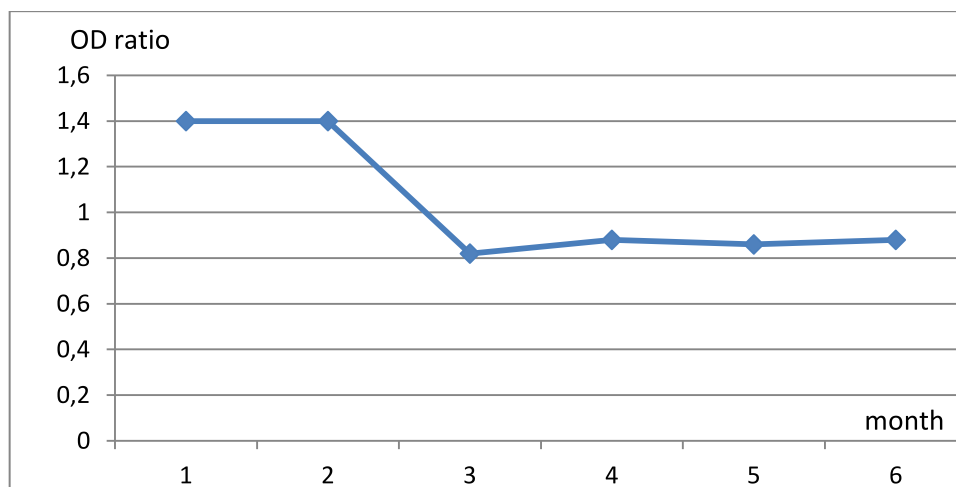
Graph 4.4. OD ratio curve during the follow-up period of re-infection patient (patient number 362)

Illiterate woman, 37 years old. Her husband had many sexual partner, and mental illness. She got family and sexual violence. She was diagnosed having trichomoniasis by clinical examination and *T.vaginalis* was observed with microscopy. At the 7th day of metronidazole 500mg b.i.d treatment, she came to our laboratory for follow-up. The microscopy, culture and multiplex PCR test did not showed *T. vaginalis* eventhough slight cervicitis and white discharge were still noted. ELISA test shown that the ratio of OD Pa/NC was 1.65. At the first time of re-examination, *T. vaginalis* was not found. During this time, she had no sexual intercourse. Two and three months later,

trichomonads were again demonstrated with microscopy and culture. *T. vaginalis* and *U.urealyticum* were also identified by multiplex PCR (Figure A.5). She was treated again with metronidazole 500mg two time a day for 10 days. At the 4th monitoring, trichomonas were still seen with *M. hominis* and *U. urealyticum*. We encouraged her husband to have a standard treatment and avoid sexual intercourse untill re-examination. Her husband's antibody titer ratio was 0.60. In final re-examination (after 5 months), *T. vaginalis* was eradicated, and OD ratio of Pa/NC of the patient was 0.65.

WB analysis shows strong antibody reaction with *T. vaginalis* antigen, with different pattern in each follow-up point (figure 4.2).

Case 3



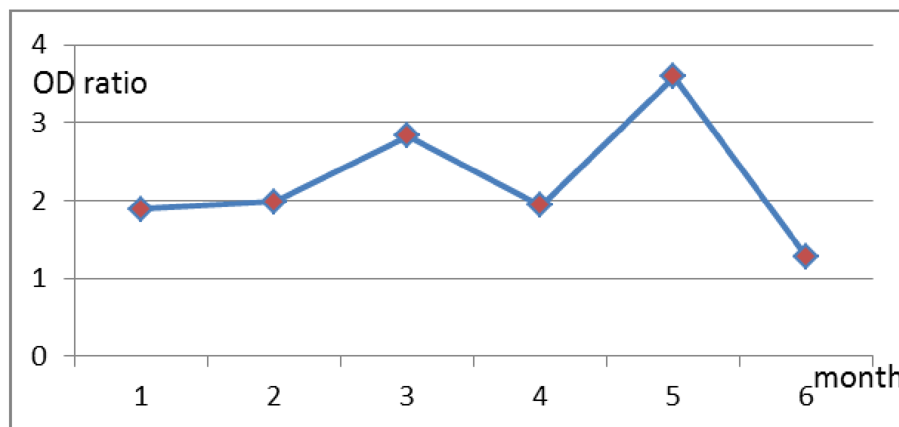
Graph 4.5. OD ratio curve during the follow-up period of patient number 84

A 49 year - old woman with white, abundant discharge, and cervicitis . *T.vaginalis* were observed by microscopic examination and culture. MultiplexPCR shown trichomonad with *M. hominis, U. urealyticum*. Her husband had a full treatment regimen and avoid sexual intercourse in the first month of treatment. He didn't present urethrisis symptoms and urine culture did not show *T.vaginalis*. ELISA test of his serum had a OD Pa/NC of 0.114/0.139.

After taking two tablets of metronidazole 250mg, she got serious rashes on skin, and she told that she had a history of metronidazole allergy. She was treted with traditional medicine (vaginal washing with greentea solution) several time per day for 1 week replaced 5-nitroimidazole treatment. Supprisingly, her symptoms improved during 5 month- monitoring time. *T.vaginalis* was not found by all of microscopic examination, culture and PCR testing. The ELISA antibody OD ratio decreased gradually and was lower than 1.0 at the second monitoring time. The recovering was also evident by gynecological examination.

On WB most antibody to *T.vaginalis* disappeared during the follow up time with some remaining reaction with antigens of molecular weight of 84-115kDa.

Case 4



Graph 4.6. OD ratio curve during the follow-up period of a clinically resistant case (Patient number 69).

Case 4. A 49 year - old, married, perimenopausal woman living in Quangbinh Province (Northern Centre of Vietnam).

She came to our clinic in December 2010 after having been treated with metronidazole by several physicians over a period of ten years for trichomoniasis disease. Her main complains was the presence of malodor,

yellowish- green discharge. The gynecological examination vagina and cervix appeared intensively erythematous and a yellowish- green frothy discharge were noted. The pH of vaginal fluid was 6. On microscopic examination, an abundance of PMNs and Tv were observed; no clue cells or yeasts were present. Multiplex PCR shown *T. vaginalis* co-infection with *M. hominis* and *U. urealyticum*. Oral metronidazole, 7-day regimen, 500 mg b.i.d. in 7 days was prescribed. *T. vaginalis* were still observed on microscopic examination. Since the patient had sexual intercourse during this period (possible reinfection) the same oral treatment regimen accompanied by local vaginal metronidazole was given but *T.vaginalis* was still identified after treatment. She was then treated with tinidazole 2g single dose, tinidazole 2g two day regimens without success even though she stopped sexual intercourse during the next two follow – up periods. Finally, the infection was cured with tinidazole 1g/daily for 7 days. Multiplex PCR before treatment and during 5 months follow – up showed the following behavior of positivity: 0: *TvMhUu, 1: TvMhUu*, 2: *TvUu,3: TvUu,4: Tv* 5: all negative.

5. DISCUSSION

During the study period from September, 2010 to June of 2012, the sample included 249 symptomatic women, 534 asymptomatic women, 38 healthy men, 8 male partners of trichomoniasis patients. In addition, 50 sera samples of children 2-10 year old, presumably not having been exposed to trichomoniasis, were used as negative controls. The ELISA assay was previously tested with a total of 46 sera from patients (selected from symptomatic and asymptomatic women groups) affected by trichomoniasis in order to set up the technique.

5.1. Epidemiology of trichomoniasis

There are many techniques to diagnose *T.vaginalis* infection. Among them, direct microscopy is really a practical and economical method with sensitivity from 60% to 75%[154,155] depending on different reports. However, Fernando in Sri Lanka (2011) showed that the sensitivity and specificity of direct microscopy can be up to 95.83% and 100%, respectively, in comparison to culture[156]. In the McCann's investigation (1974), 22.3 % of cases would have been missed using culture only and 13.7% would have been missed if culture not been used[89].

In present study, we should use microscopic examination to select the trichomoniasis patients for follow up visits, therefore the vaginals samples are examined up to 15 minutes, to improve diagnosis. The prevalence of trichomoniasis diagnosed by microscopic examination in symtomatic and asymptomatic women groups were 19.3% (48/249, 95% CI = 14.6% - 24.8%) and 0.7% (4/534, 95% CI = 0.18% - 1.8%), respectively.

Using the same methodology, wet mount preparation, the prevalence of trichomoniasis varies largely from a maximum in Northern Central of Vietnam of 5.21% (Le VT, 2004)[157] to Mid-Central of Vietnam 2,38% (Nguyen K M, 2009)[158] to a minimum in Highland of 0.3% (Cao TTB, 2006)[159]. In another study of Ly Van Son (2008) the prevalence of *T.vaginalis* infection was 0.98% in STDs clinic at Hue City[160], while the prevalence of *T. vaginalis* in Nigeria was 0.37% in study of Omoregie et al. 2010[161]. There are also variable prevalence of *T. vaginalis* infection in other countries, for example: in Vientiane, Lao 3.7%[162], in France 3.1%[163] (Lefrevre 1988), in Turkey 8.69% (Tamer 2009)[164], in Iraq 2.4%[165] (2011) and in Bakau, Gambia 32% (1984)[166].

The study of Sumadhya in Sri Lanka (2012) showed that the prevalence of *T. vaginalis* infection was higher in women with low educational level than women with high educational level[1] as it was showed in this study while Annang' study in USA (2011) showed that educational status was not uniformly protective against STIs for black and white females in US[167]. In our study, the prevalence of *T. vaginalis* infection in low educational level of women was higher than it in high educational level.

The prevalence of *T.vaginalis* infection (18.3%) in women with unsafe sex behavior was significantly higher than in women with safe sex behavior (3.2%). Many other surveys revealed the same finding that, multiple sexual partners and not using condoms during sexual acts may are increasing *T. vaginalis* infection[168],[169]. The difference was also significantly among geographic areas (urban vs. rural) probably because of the difference in lifestyle between the city and countryside.

The mean ages was similar in both groups: 37 year for symptomatic women and 38 year for asymptomatic women, and 37 in trichomoniasis patients which was similar in a survey of Dan M. et al. (1996) in USA[151], but higher than many other study and reports from CDC (2010)[131]. Jones in USA (2007) revealed that women aged 23–25 years were nearly three times more prone to seek for medical care in comparison with women aged 14–17, after adjustment for randomization group[170]. Older age seems to be a risk factor of *T.vaginalis* infection as reported by several other authors[171,172]. The increased prevalence of infection in older women may indicate a long standing infection that does not spontaneously resolve and that is likely missed by screening programs focused on younger women[173]. In addition, Bowden et al. (1999) showed the prevalence of *T. vaginalis* infection was significant increasing with age[174].

All over the world, there are very rare reports of trichomoniasis in menopausal women because the frequency and number of sexual intercourses decreased significantly as the age and the menopausal status advance. The study performed by Spinillo et al. (1997) showed an lower odds ratio (OR) 0.53, (95% CI, 0.37–0.75) of postmenopausal women with *T. vaginalis* or *C. albicans* infection, or bacterial vaginosis, or mixed infection versus women in reproductive age[80]. In present study, there are 4 (8.7%) menopausal trichomoniasis patients to compare with 10% (2/20) menopausal trichomoniasis patients in the study of Akarsu in Turkey (2006)[175].

In our patients, the main reasons leading them to attend the gynecological clinic were malodorous vaginal discharge (73.9%) and the profuse vaginal discharge (60.9%). The typical signs of trichomoniasis were yellowish-green frothy discharge (54.3%) and strawberry cervix (30.4%). Reported rate of the above mentioned symptoms were higher than other studies: strawberry cervix

5%-10% (Carr P.L. 1998)[155], yellowish-green frothy discharge 47%, abnormal cervix 16% (Sumadhya. et al. 2012)[1]. Dan et al. (1996) reported that the malodor vaginal discharge, profuse vaginal discharge, vaginal erythema, cervicitis were common both of acute and chronic cases[152]. In addition, Heine et al (1993) showed that trichomoniasis was commonly a chronic disease and up to one-third of asymptomatic women will develop symptomatic infection within 6 months[176]. In our study, case number 1 (graph 4.3) described asymptomatic patient developed symptomatic infection after 6 months. Otherwise, since 1980, the study from Fouts et al (1980) already showed that a purulent, frothy discharge is indeed a characteristic of trichomonal vaginitis, but if it is used as the sole diagnostic criterion, 88% of women with trichomonal vaginitis will not be identified and 29% will be erroneously diagnosed as infected[93].

In general, the clinical features of this disease are variable. Therefore, a combination of clinical and microbiological examination should be performed.

5.2. Seroepidemiology of trichomoniasis

5.2.1. Comparison of anti – *T. vaginalis* specific antibody reaction between trichomoniasis patients and different study groups

This is the first study in Vietnam using ELISA assay to find human sera IgG antibody to *T. vaginalis* antigen. Immunological data, as shown in the graph 4.1, is a useful technique with good sensitivity and specificity when direct examination is used as standard diagnostic method. Direct examination can be the better standard for ELISA assay than culture and PCR technique because culture and PCR detect *T. vaginalis* in very early stage of the disease. Data of mean OD measurement from study groups were statistically different,

confirming the usefulness of ELISA assay in diagnosis of *T. vaginalis* infection (table 4.3).

Results obtained demonstrate that ELISA assay are useful for better understanding the mechanisms involved in host - parasite immunological relationships.

5.2.2. Seroprevalence of *T. vaginalis* antibody in subgroups of study

From data from Table 4.5, the anti-*T. vaginalis* seropositive rate was higher than those diagnosed by microscopic examination, showing that the sensitivity of ELISA assay using the whole cell of *T. vaginalis* as antigen to detect IgG antibody was higher than microscopy examination.

Epidemiologic data based on direct examination and ELISA yielded similar results with statistically significant differentts between subgroups (symptomatic vs. asymptomatic, urban vs. rural, unsafe sex vs. safe sex ...).

ELISA assay is usefull test for seroepidemiological screening in community to indicate the risk factor of STDs transmission.

5.3. Follow up of selected patients

The result of evaluation of sera antibody titer during the five months of follow up period was given on table 4.6. There are two statistically different trending line of human IgG antibody titer to *T. vaginalis* in recovering and unrecovering or re-infection groups follow-up since the line of is going down after 4-5 months in the group of recovering patients while those from the unrecovering/reinfection patients maintain a high level of antibody during all the period. This can be considered as a good marker of persistent infection.

In addition, in recovering patients, the symptoms improved gradually over time, and at the third follow-up 100% of clinical symptoms show improvement. The existence of high level of antibody and clinical symptom in

unrecovering/reinfectious patients during the follow up time suggest a close relationship between clinical symptoms and antibody level.

Immunobloting confirms the correlation between clinical symptoms, *T. vaginalis* identification, ELISA antibody titer and specific reactive antigen. All sera, both male and female recognize common antigen molecules of about 100kDa MW (84 - 115kDa). This is consistent with previous finding (Addis et al. 1999, Wos SM et al. 1986, Gaber et al 1986)[65,66,177] and makes the search for common immunogens particularly appealing with possible application for a more sensitive serologic test or as a possible vaccinogen and for studies of pathogenicity.

In general, eventhough, most of antibody disappeared during the recovery time, there were the persistance of antibodies weighting 84 -115 kDa in both of trichomoniasis patients and their partners.

In present report, we also show some trichomonas vaginitis cases in Vietnam that are usefull for understanding the complex interaction of the parasite with its host and the effect of treatment.

Case 1 might be a recurrent exposure with *T. vaginalis* because her husband had several sex partners, and he did not agree to have treatment. Even though, *T. vaginalis* were not identified during-up clinical symptoms of trichomoniasis persisted after several metronidazole treatment. The infection occuring six months later can be explained as persistence of metronidazole-resistant *T.vaginalis* or, most probably with a reinfection from her sexual partner. Molecular analysis with the isolated strains may help to clarify this issue.

Case 2 appears as a typical example of reinfection. *T.vaginalis* of the initial infection were quickly eradicated with standard metronidazole treatment but the patient was again reinfected, after having sex, by metronidazol-sensitive

strains requiring new treatment. WB analysis showed a slight difference in antibody pattern during the follow-up period probably due to reinfection. This and the previous case confirm the lack of protection from repeated infections.

In case number 3, a metronidazole allergic patient, the microbiological and clinical cure was obtained by vaginal washing with greentea infusion and, if confirmed, may open interesting application of traditional Vietnamese medicine to treatment of trichomoniasis.

Otherwise, case number 4 was unsuccessfully treated with metronidazole and tinidazole at limited dosage but was finally treated with tinidazole for a longer period. The re-infection could be excluded in this case. Metronidazole resistance is defined by patients' failure to clear infection after one standard course of treatment (Xiao J. C. et al. 2006)[141], and refractory cases of trichomoniasis, defined as cases in which two standard courses of treatment fail to cure (Cudmore S. L.et al 2004)[74]. The case 1, resistant to metronidazole and with limited sensitivity to tinidazole might be interpreted as a clinically refractory trichomoniasis.

This, as fare as we know, is the first case of metronidazole resistance reported from Vietnam.

Despite the limited number of patients involved, this study showed a wide spectrum of clinical features of *T. vaginalis* infection and host immunoresponse and documented the probable presence of metronidazole resistance in Vietnam

CONCLUSIONS

The prevalence of trichomoniasis diagnosed by microscopic examination in symptomatic women and asymptomatic groups were 19.3% (42/243, 95% CI = 12.8% - 22.7%) and 0.7% (4/534, 95% CI = 0.18% - 1.8%), respectively. Clinical features of *T. vaginalis* infection showed a wide spectum. The most prevalent symptoms were vaginal erythema, malodorous vaginal discharge, profuse vaginal discharge, cervicitis, and yellowish-green frothy discharge. There was 8.7% asymptomatic patients. Co-infection with *M. hominis*, *U. urealyticum* and both of them has been recorded in 39.1%, 23.9% and 28.3%, respectively. There were only 8.7% infected *T. vaginalis* alone.

ELISA essay yielded high sensitivity and specificity. The sensitivity of ELISA assay was higher than microscopy examination. This test may indicate the risk factors that increases the risk of STDs transmission.

The seroprevalence from general population were found 18.9% in women and 8.7% in men. The seroprevalence were 31.3% in symptomatic women, 13.3% in asymptomatic women. The seroprevalence was 14% in safe sex behavior women to compare with 22.7% in unsafe sex behavior women. There were 7.9% seropositive from sera of healthy men and 12.5% seropositive from sera of men partner of trichomoniasis women.

Serological follow-up by ELISA showed the trending line of sera *T. vaginalis* IgG antibody going down after 4 - 5 months in the group of recovered patients; while those from the unrecovered or re-infection patients kept the high level of IgG antibody, a marker of infection persistence.

Detection of specific antibody response in sera could be considered as a good marker for therapy success.

Results from Western blot analysis showed significant correlation with those from ELISA assay and clinical symptoms during the course of follow-up periods.

There were the persistence of antibody to *T. vaginalis* antigen 84 -115kDa in men partners and recovery patients.

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Appendix 1

INFORMATION COLLECTION FORM OF TRICHOMONAS RESEARCH IN VIETNAM

1. **Name:** **Code number**:
2. **Age:**
3. **Sex:**
4. **Profession:**
5. **Address**: 5.1. Urbal 5.2. Rural 5.3. Highland
6. **Phone number**:
7. **Education degree**: 7. 1. No education
   7. 2. Primary school
   7. 3. Secondary
   7. 4. Hight school
   7. 5. College or higher

8. **Marital status** : 8. 1. single
8. 2. marriage
8. 3. separate
8. 4. divorced
8. 5. widow
8. 6. anything else :……

9. Profession of husband/partner:

10. Gynecological history:

11. **Reason to go for medical exam**

11.1. Profuse vaginal discharge

11.2.White discharge

11.3. Purulent discharge

11.4. Yellowish-green frothy discharge

11.5. Lower abdominal tenderness

11.6. Vulvovaginal itching and soreness

11.7. Dysuria (pain during urination)

11.8. Dyspareuunia (pain during sexual intercourse)

11.9. Other reasons:

12. Clinical symptom

12.1. Vulvar/ vaginal erythema

12.2. Cervicitis

12.3. Strawbery cervix

12.4. Vaginal malodor

12.5. Profuse vaginal discharge

12.6. Yellowish-green frothy discharge

12.7. White discharge

12.8. Purulent discharge

13. Direct exam

13.1. pH:

13.2. Clue cell:

13.3. PMNs:

13.4. *T.vaginalis*: Positive □ Negative □

13.5. *Candida*: Positive □ Negative □

14. Culture *T.vaginalis*: Positive □ Negative □

15. MultiplexPCR *T. vaginalis* □ *M. hominis* □ *U. urealyticum*□

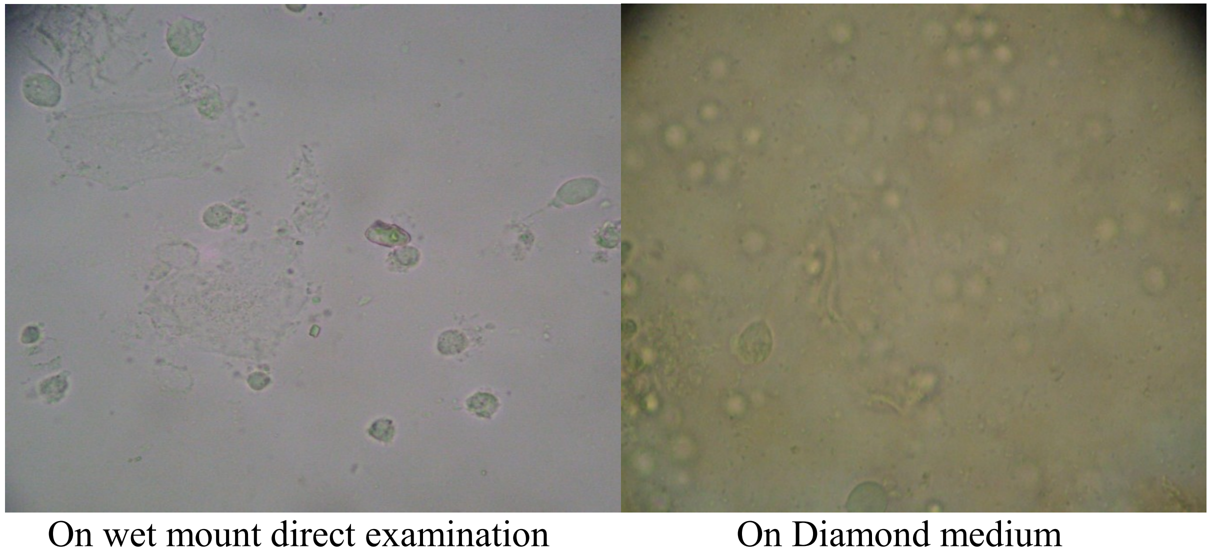
16. ELISA test assay: OD ratio of Pa/NC

Hue, date…./…./20…
Interviewer

Note: *+ Code number/1 to 5: first to fifth time of follow up visits*

+ Code number/ C: men partner of trichomoniasis women

Appendix 2



On wet mount direct examination On Diamond medium

Figure A1. *T. vaginalis* on microscope

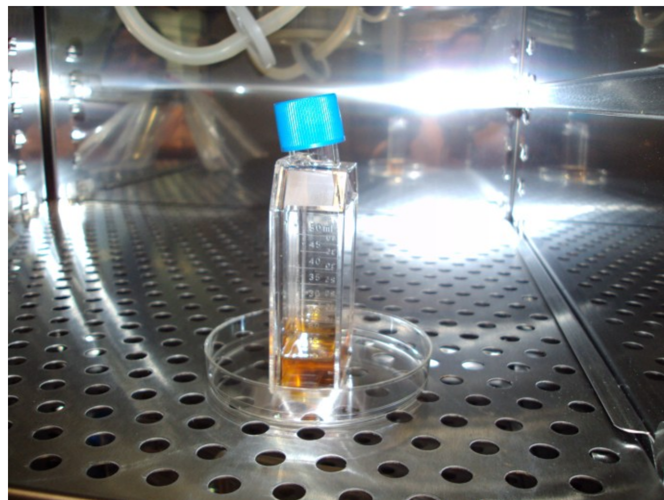
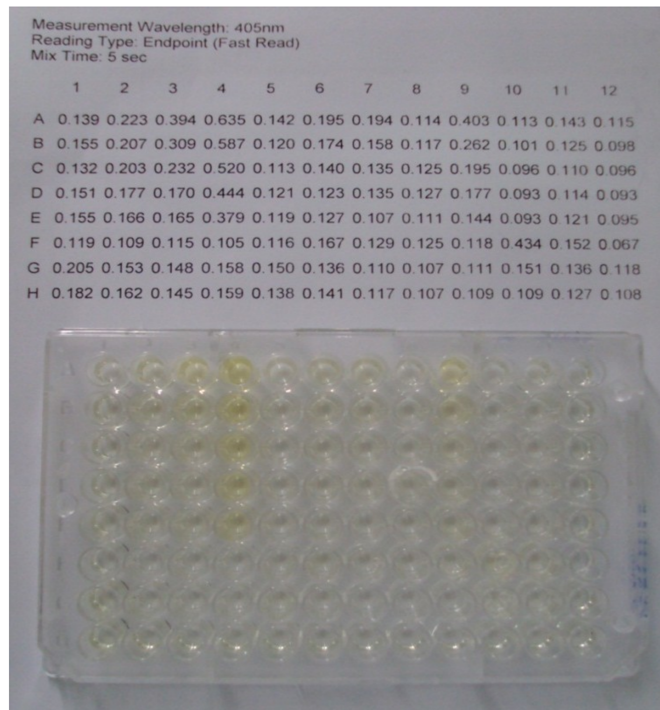


Figure A 2. Axenic culture of *T. vaginalis* on Diamond medium



Measurement Wavelength: 405nm
Reading Type: Endpoint (Fast Read)
Mix Time: 5 sec

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.139	0.223	0.394	0.635	0.142	0.195	0.194	0.114	0.403	0.113	0.143	0.115
B	0.155	0.207	0.309	0.587	0.120	0.174	0.158	0.117	0.262	0.101	0.125	0.098
C	0.132	0.203	0.232	0.520	0.113	0.140	0.135	0.125	0.195	0.096	0.110	0.096
D	0.151	0.177	0.170	0.444	0.121	0.123	0.135	0.127	0.177	0.093	0.114	0.093
E	0.155	0.166	0.165	0.379	0.119	0.127	0.107	0.111	0.144	0.093	0.121	0.095
F	0.119	0.109	0.115	0.105	0.116	0.167	0.129	0.125	0.118	0.434	0.152	0.067
G	0.205	0.153	0.148	0.158	0.150	0.136	0.110	0.107	0.111	0.151	0.136	0.118
H	0.182	0.162	0.145	0.159	0.138	0.141	0.117	0.107	0.109	0.109	0.127	0.108

Figure A3. ELISA assay: 1A negative control, 2B Positive control, 12H White well of PBS

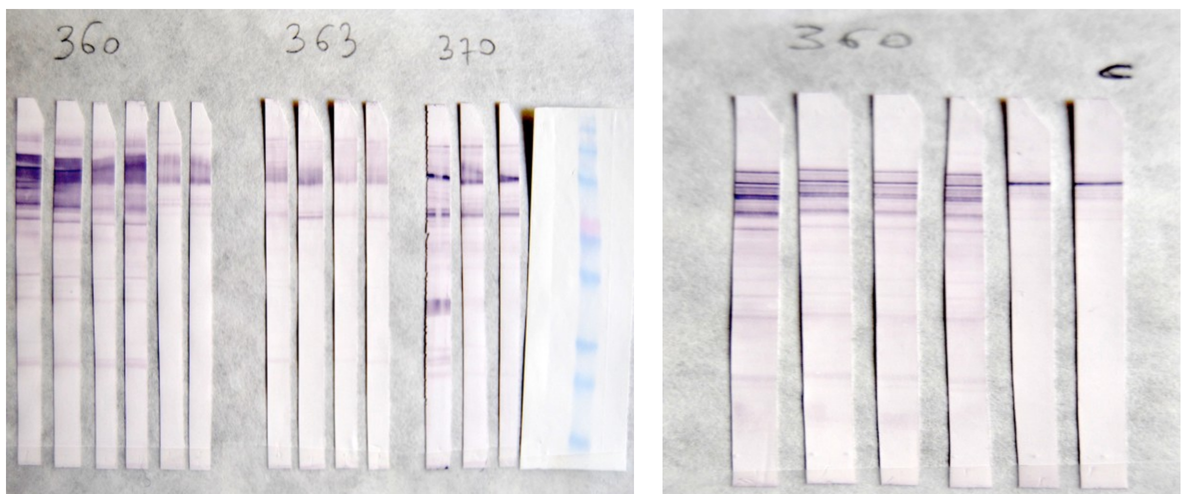


Figure A4. Wester blotting analysis of different reaction of recovery patients and her partner (360C) during the follow up time.

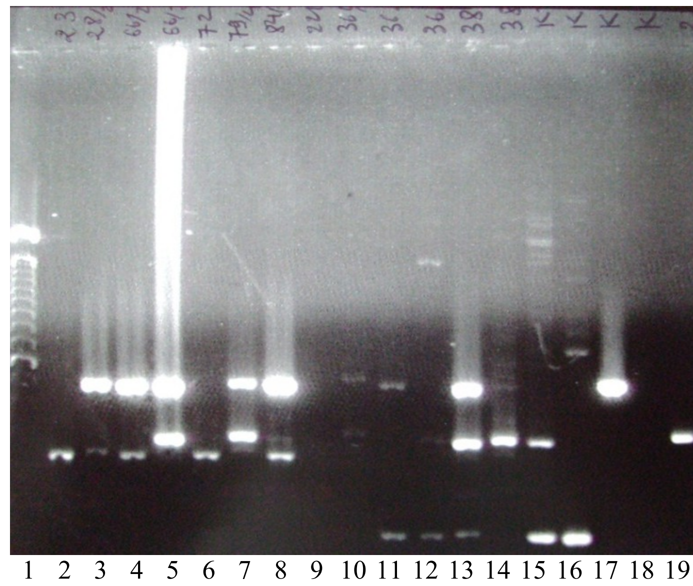


Figure A 5. Lane 1: 100 bp DNA ladder, Lane 3, 4, 5, 6, 8, 9: recovery patient, Lane 7: recurent exposure patient (case number 2), Lane 11: re infection patient (case number 3), Lane 15: Control of *Tv* (102bp) *Mh* (334bp), Lane 16. Marker of *Tv* (102bp), Lane 17 Marker of *Uu* (541bp).

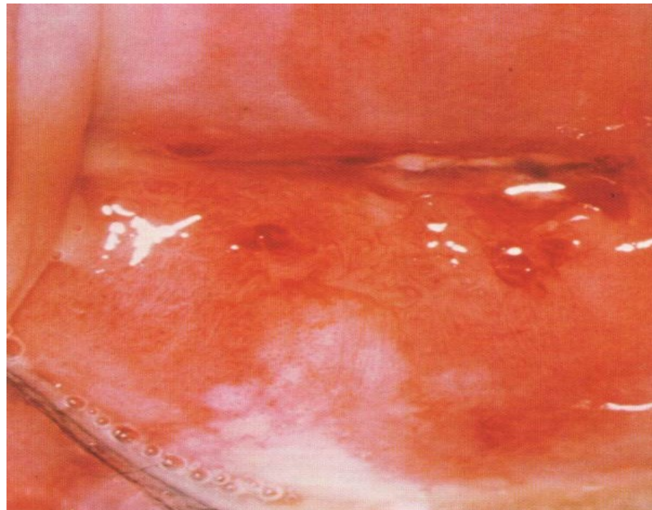


Figure A6. Yellowish green forthy vaginal discharge

Appendix 3. **MEDIA AND BUFFERS**

PROTOCOL OF PREPARATION OF DIAMOND MEDIUM

Regent

Yeast extract	**20g**
Casein pancreatic Peptone	**40g**
Maltose	**10g**
L-cystein	**2.24g**
Ascorbic acid	**0.4g**
K2HPO4	**1.6g**
KH2PO4	**1.6g**
fenol red	**0.02g**
H20	**2 liters**
pH	**6.6**

Procedure

a. Dissolving Yeast extract, casein pancreatic Peptone, Maltose, L-cystein, Ascorbic acid, K_2HPO4, KH_2PO_4, fenol red in 2000ml distilled water, and sterilizing by autoclave at 15lbs pressure and 121^0C for 30 minutes.

b. Bringing the medium to cool at room temperature then adding 1,000UI/ml Penicillium, 100µg/ml Streptomycin, 25µg/ml Fluconazole, and add 10% (200ml) Foetal bovin serum when using.

c. Medium can be refrigerated at 4^0C for store.

BUFFERS

1. AP-Buffer pH 9.5

Tris base	0.1M (6.055g/500ml
NaCl	0.1M (2.922g/500ml)
$MgCl_2$	0.005M (0.508g/500ml)
Distilled water	500ml

2. Phosphate Buffered Saline (PBS) 1X, pH=7.4

NaCl (137M)	8g
KCl (2,7M)	0.2g
$Na_2HPO_412H_20$ (4,3M)	2.9g
KH_2PO_4 (1,8M)	0.2g
Distilled water	1000ml

Appendix 4

List of trichomoniasis patients

No	Patient Code	Name	Age	Address	Education level	Marital status
1	28	Tran T X	43	rural	low	widow
2	66	Ho T T	35	rural	low	widow
3	68	Nguyen TMS	23	urban	high	married
4	69	Tran TL	48	rural	high	married
5	79	Tran TH	52	urban	high	married
6	81	Tran T M N	44	urban	low	married
7	84	Nguyen T H	49	urban	low	married
8	227	Hoang T D T	30	rural	high	widow
9	360	Nguyen T H T	28	rural	high	married
10	361	Nguyen T T N	23	rural	high	divorced
11	362	Tran T S	37	urban	low	married
12	363	Luong T K	60	urban	high	married
13	85	Nguyen T T	49	rural	low	widow
14	365	Ho T B D	27	urban	low	married
15	366	Nguyen T L	50	rural	high	married
16	367	Nguyen T T	39	rural	low	married
17	368	Nguyen T H	53	urban	high	married
18	369	Nguyen T P T	32	urban	high	married
19	370	Dao T T	25	rural	low	divorced
20	371	Le T M	50	urban	low	married
21	372	Nguyen T H H	34	urban	high	married
22	373	Van T X	20	rural	low	single
23	374	Ton N T T	37	urban	low	married

24	961	Nguyen T T	30	urban	high	married
25	375	Tran T T	35	highland	low	divorced
26	376	Ha T P	29	urban	low	married
27	574	Tran T K O	29	rural	low	married
28	377	Duong T B L	37	urban	high	married
29	378	Tran T L	41	highland	low	married
30	379	Nguyen T T	45	rural	low	married
31	380	Tran T L	34	rural	low	widow
32	382	Nguyen T L A	29	urban	low	married
33	383	Ho T N	40	urban	low	married
34	384	Tran T T	43	urban	low	widow
35	385	Phan T D H	38	urban	low	married
36	386	Nguyen T A H	46	urban	low	married
37	387	Le T B Y	26	urban	high	single
38	388	Ha T M T	20	rural	high	single
39	389	Hoang T M L	31	urban	high	married
40	390	Tran T T C	32	urban	high	married
41	391	Nguyen T N H	34	rural	low	divorced
42	392	Vo T H	39	rural	low	widow
43	393	Dang T T V	40	urban	high	married
44	394	Nguyen T T	43	urban	low	married
45	395	Nguyen T H	45	rural	low	married
46	397	Doan T T D	30	rural	low	married

Appendix 5

ETHIC APPROVAL

APPLICATION FOR ETHIC APPROVAL FOR IMPLEMENTATION OF SCIENTIFIC RESEARCH PROPOSAL

To: **The Chairman, Ethics and Scientific Committee of Hue University of Medicine and Pharmacy**

Name of investigator: TON NU PHUONG ANH

The title of study project: **PREVALENCE OF TRICHOMONIASIS IN HUE CITY, VIETNAM: A SEROLOGICAL STUDY**

This study is a part of my study project for my PhD thesis of Doctorate programme in Biomolecular and Biotechnological Sciences in Sassari University, Italy.

Supervisor: Prof. Pier Luigi Fiori, University of Sassari

Project objectives:

1. To estimate the prevalence of T. vaginalis in high risk and low risk population of Hue City, Vietnam by clinical, wet mount microscopic, and serological examination.

2. To evaluate the antibody response against T. vaginalis during follow-up visits and determine the kinetic of antibody disappareance in sera of treated patients.

The intended period of time for the study project:

From September 2010 to June 2012.

The participants to be enrolled:

- All individuals diagnosed with vaginal trichomoniasis by direct examination. They will be recruited from Gynecological Clinic of Hue University Hospital, Reproductive Healthcare Centre of Thua Thien Hue Province, and from general population of Thua Thien Hue Province. The male partners of the trichomoniasis women will be also included in the study.

- The women attending the Gynecological Clinic of Hue University Hospital.

- The women in community of Thua Thien Hue Province (Phu Vang District, Hue City, and Nam Dong District) participated in to this study.

- The men who visited the Parasitology Department for examination of dermatophytose and volunteer students of Hue University of Medicine and Pharmacy.

The study procedures:

The study will be carried out in order to implement the following steps:

- Providing and obtaining informed consents.
- Completing the patient history forms and performing clinical examinations.
- Collecting vaginal discharge and blood samples for laboratory testings.

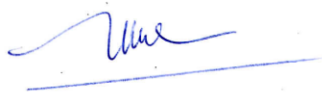
Declaration of responsibility:

- Agreement (in oral or writing) from patients will be taken before including them into study: "I am aware that any medical procedure, which can be used on patients, may bring all the potential risks to patients' health and I also know with certainly that the procedure preformed for collection of specimen will make no harm to the patients".
- The patient data, record and results of examination and laboratory analysis will be encoded, kept only for scientific purpose and not for anything else.

I confirm that the imformation contained in this application is correct and true.

Hue 21st August, 2010

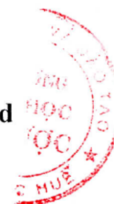
Investigator signature

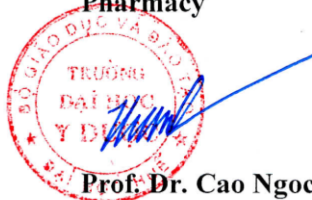


Ton Nu Phuong Anh

Approval

Ethics and Scientific Committee of Hue University of Medicine and Pharmacy





Prof. Dr. Cao Ngoc Thanh

Rector, Chairman

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other insititution of higher learning, except where due acknowledgement is made in the acknowledgements.

Name: Ton Nu Phuong Anh Sign:

ACKNOWLEGEMENT

Many people and organizations contributed to the completion of my PhD study and are too numerous to name although I acknowledge the contribution and assistance provided by everyone. Specifically I would like to express my sincere gratitude and appreciation to the following people and organizations that enabled me to study in Italy and Vietnam, and for the ideas and suggestions given to me during the research process.

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