EVALUATION OF A POLYMERASE CHAIN REACTION (PCR) ASSAY FOR THE DIAGNOSIS OF *TRICHOMONAS VAGINALIS* INFECTION

A Dissertation done towards partial fulfillment of the requirements of the **Tamil Nadu Dr. M. G. R. Medical University**, **Chennai** for the **M.D. (Branch – Clinical Microbiology)** exams to be conducted in March 2009

BONAFIDE CERTIFICATE

This is to certify that the work presented in this dissertation titled **"EVALUATION OF A POLYMERASE CHAIN REACTION (PCR) ASSAY FOR THE DIAGNOSIS OF *TRICHOMONAS VAGINALIS* INFECTION"** done by **Dr. Hema Paul**. She independently reviewed the literature and carried out the techniques used. The work was carried out in the Department of Clinical Microbiology, Christian Medical College, Vellore, under the guidance of **Dr. Rajesh K.**, Professor of Microbiology. We certify the thesis as the bonafide work of **Dr. Hema Paul.**

Dr. Rajesh. K, M.D., PhD
Professor (Guide)
Clinical Virology
Dept. of Clinical Microbiology
Vellore
Christian Medical College, Vellore
Tamil Nadu, 632004

Dr. Mary Mathews, MD
Professor and Head
Dept. of Clinical Microbiology
Christian Medical College,
Tamil Nadu, 632004

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INTRODUCTION

1.0 INTRODUCTION

Trichomonas vaginalis is a protozoan parasite and etiologic agent for Trichomoniasis, a sexually transmitted disease (STD) (40). Trichomoniasis is a common non-viral sexually transmitted disease (114). The global incidence of trichomoniasis is 174 million annually (114). In South and South East Asia, 76.5 million new cases are reported annually (114). Symptomatic trichomoniasis is more common in women than in men. Trichomoniasis is most prevalent during the peak years of sexual activity (102). The route of transmission is almost exclusively by sexual intercourse. It has been estimated that 10-50% of *Trichomonas vaginalis* infection in women is asymptomatic (67, 90).

Trichomoniasis has a wide spectrum of symptoms ranging from inflammation in the form of vaginitis, urethritis to malodourous frothy discharge alone with asymptomatic carrier state (24, 102). The discharge is frothy yellow or green and mucopurulent (85). About 2% of infected patients present with "strawberry cervix" (24). Trichomoniasis is associated with serious complications. These include pyosalpinx, endometritis, and premature rupture of membrane, preterm labor and low birth weight (34, 70). *Trichomonas vaginalis* is associated with non gonococcal urethritis and prostatitis in men (48).

There are several epidemiological studies done in India which shows that trichomoniasis is a major problem. Hospital based studies done in India showed that the prevalence of *Trichomonas vaginalis* as high as 61.7% in women attending STD clinic while as low as 6.7% among women with reproductive tract infections (1, 93).

Community based studies among women from different parts of India had shown prevalence for *Trichomonas vaginalis* varies from 13 - 34.3% (17, 83). A community based study among the spouses of women with *Trichomonas vaginalis* infection showed a prevalence of 47.5% (51). In a hospital based pregnant women population of Tamil Nadu the prevalence was found to be 6% (67).

It is known that sexually transmitted infections (STI) facilitate the transmission and acquisition of other STI's including human immunodeficiency virus (HIV) (65). Measures like aggressive STI prevention, testing and treatment reduce the transmission of HIV. In studies done among the HIV seropositive and HIV seronegative women the prevalence of trichomoniasis was 9.4-29.5% and 8.2%-23.4% respectively in a group of female sex workers (19). The incidence of trichomoniasis also found to be high among women compared to men among HIV infected individuals (42). Among pregnant women infected and uninfected with HIV virus higher incidence of Trichomoniasis was present in seropositive than seronegative women (101).

Trichomoniasis is a non notifiable infection and hence no public health programs have been directed towards controlling the infection (91). This disease plays an important role in social, medical and economical areas of the society. Majority of the women with trichomoniasis have been asymptomatic (67, 90) and in the absence treatment, infection is thought to persist for long periods (16).

Currently there are different techniques available for the diagnosis of trichomoniasis. The various clinical specimens that can be used for the diagnosis of infection include urine, vaginal fluid, semen and endo-cervical smear from women where as urethral discharge, prostatic secretions or urethral scrapings may be used for diagnosis in men (102). The most commonly

used methods for the diagnosis of trichomoniasis infection are wet mount preparation and culture (90). Both these methods rely on visual detection of viable trichomonads with their characteristic motility (44). The wet mount preparation has shown to have a sensitivity of 35-82% and a specificity of 95% (62, 91). The advantage of the wet preparation is that, it is inexpensive and rapid (22) while the disadvantage is that the sensitivity depends on the experience of the performer (62). There are different staining methods also in use for the diagnosis of the *Trichomonas vaginalis*. Papanicolaou (Pap) smears have shown a sensitivity of 61.4% (53). Other staining methods used are Giemsa, Romanowsky and Acridine orange (63). Though the above mentioned tests are rapid with sensitivity as that of wet mount preparation, they require skilled personnel, financial resource as well as good equipped laboratory (102). Broth culture is considered as a gold standard for diagnosis of trichomoniasis (17). There are several culture media available for the cultivation of *Trichomonas vaginalis*. The reported sensitivity for the culture is 85%-97% and the specificity varies from 94% to 100% (28, 62,103). The drawbacks of culture are its cost and duration of incubation is required for the growth of the organism. This period can be prolonged to 2-7days (24). Hybridization assay also has been also used for the diagnosis and has a sensitivity of 88-91% and a specificity of 100% (115). Even though this is a rapid test it requires heat block special processor which makes it expensive and suitable only for reference laboratories.

Among the newer tests, a nucleic acid amplification method, like polymerase chain reaction (PCR) seems to be more practical in diagnosing *T.vaginalis* infection. PCR requires only DNA of viable or non-viable organism in very low concentrations (102). The sensitivities of various primers designed ranges from 85-100% (91). As compared to culture the results are available faster (62).

There is insufficient information from India on the prevalence of *Trichomonas vaginalis* especially among the HIV infected individual. The data on molecular diagnosis and its comparison with the widely available culture and wet mount preparation is lacking from India. Hence, this study is being undertaken to evaluate the role of polymerase chain reaction (PCR) as compared to wet mount and culture in the diagnosis of Trichomoniasis and also to look at the frequency of infection among HIV infected women.

The hypothesis of the study was that the prevalence of trichomoniasis in HIV infected women is higher and PCR is a better alternative method for the diagnosis of *trichomonas vaginalis*.

AIM AND OBJECTIVES

2.0 AIM AND OBJECTIVES

AIM

To standardize and evaluate a Polymerase Chain Reaction (PCR) for the diagnosis of trichomoniasis.

OBJECTIVES

1. To standardize Polymerase Chain Reaction (PCR) for the diagnosis of *Trichomonas Vaginalis* in culture from clinical samples

2. To evaluate the performance of the PCR in diagnosing trichomoniasis in the hospital setting.

3. To determine the prevalence of trichomoniasis in Human Immunodefiency Virus (HIV) positive women.

REVIEW OF LITERATURE

3.0 REVIEW OF LITERATURE

3.1 HISTORY AND EVOLUTION

Donné first discovered and named *Trichomonas vaginalis* in 1836 after finding the organism in genital secretions of both women and men (104). *T. vaginalis* Pathogenicity was initially thought to be non-pathogenic as majority of infected patients were asymptomatic (104). *T.vaginalis* is a parabasalid protozoan that branched before Kinetoplastids and other protozoa which has mitochondria. *T. vaginalis* lacks mitochondria. It uses hydregenosome fermentative carbohydrate metabolism, where hydrogen is used as an electron acceptor.

3.2 CLASSIFICATION:

The taxonomic classification scheme of *T.vaginalis* was given by Dyer (91). According to this

Protozoa with "9+2" flagellum, fall into the

Phylum: Zoomastigina.

Class: Parabasalia - presence of parabasal body, [Golgi associated Kinetosomes, axostyle and undulating membrane]

Order: Trichomonadila

They have 4-6 flagella which are free or attached to undulating membrane and they do not have true cystic form.

3.3 GENERAL MICROBIOLOGY:

Among all the Trichomonads, *T.vaginalis* is the most widely studied parasite. The size and shape of this protozoan is variable, the average length and width being 10 & 7 micron respectively (39). The protozoan parasite is pear shaped or oval in axenic cultures (11). It assumes an amoeboid shape when attached to the vaginal epithelium (35).

It is a flagellated protozoan with five flagella, four of which are located in the anterior part of the parasite while the fifth flagellum is incorporated within the undulating membrane supported by non contractile costa (11,109). The parasite has a characteristic quivering motility imparted by the flagellum and the undulating membrane (39). During unfavorable growth conditions, *T. vaginalis* can round up and internalize the flagella. There are some who believe these forms could be pseudocysts, but it is more likely to represent degenerate forms of *T. vaginalis* as there are no reports of these giving rises to normal motile forms (27).

The cytoskeleton of *T. vaginalis* is composed of tubulin and actin fibers. Investigators have used monoclonal antibodies to the tubulin molecule and found that the axostylar tubulin reacted with both sheep and pig brain tubulin (79). The nucleus is surrounded by a porous nuclear envelope and is situated at the anterior part of the parasite. The axostyle arising at the nucleus divides the parasite into two parts longitudinally and terminates at the sharp point at the posterior end of the parasite. The axostyle helps the parasite to adhere to the vaginal epithelial cells (39).

Granules called the hydrogenosomes are present in the paracostal and para-axostylar area (91,109). These are visible under microscope if the organism is alive. Glycogen granules are also present and can be visualized under electron microscope (109).

3.4 REPRODUCTION AND LIFE CYCLE

Trichomonas vaginalis has a poorly understood life cycle. The protozoan parasite is found to exist only in the trophozoite form There are varying developmental stages of trichomonas namely round forms which divide by amitotic budding division and small flagellated forms which divide by longitudinal binary fission and their nuclear membrane remain intact.
The process of division begins with duplication of selected loco motor organelles and the development of two attractophores on either side of the nucleus which are the poles for division. Chromosomal microtubules develop from the attractophores which grow towards and into the nucleus and attach to the centromeres of chromosomes. The paradesmose (extra nuclear spindles) elongate allowing the daughter cells to separate and develop the missing organelles (2).

3.5 EPIDEMIOLOGY:

Trichomonas vaginalis infection is found to be prevalent globally with no seasonal or climatic variation. The overall estimated global prevalence of cases of Trichomoniasis is about 174 million (114). The annual incidence of infections with *Trichomonas vaginalis* is 5 million in United States (65) and 76.5 million in South Asia and South East Asia (114).

A study done in Baltimore (USA) among women attending STD clinic which showed 26% prevalence of Trichomoniasis (99). In another study based on race or ethnicity prevalence of *Trichomonas vaginalis* was reported as 23-51% between Africans-Americans, which is about

1.5-4 times more than other racial groups (99). In India, there are fewer studies done on *T. vaginalis*. Overall community based prevalence of *T. vaginalis* among women varies from 0.8 – 34.3 % (17, 110) while the prevalence among married women varies from 4.3 – 27.4 % (83, 98). The prevalence of *T. vaginalis* in women attending sexually transmitted disease (STD) clinic varies from 5.9 – 17.2 % (20, 41, 49) while women attending gynecological out-patient department (OPD) from 0.4 – 26 % (13, 43, 78) and antenatal clinic from 3.8 – 15.7 % (67, 78, 95).

The prevalence of trichomoniasis among women by race in United States is highest (23-51%) among the African American women (99). A Study done in USA among 216 HIV infected women, using cervico-vaginal lavage 25.2% were positive for *T.vaginalis* while 49.3% were found to have bacterial vaginosis (45).

In another study done in the rural areas of Eastern Zimbabwe blood clots of 5521 women were taken to study co-infection with HIV and trichomoniasis. 516 (9.9%) were positive for *T.vaginalis* and among these 208 (40.3%) were positive for HIV while the remaining were negative for HIV (66).

3.6 MODES OF TRANSMISSION:

Human beings are the only natural host for *T.vaginalis*. Trophozoites are the infective forms and they are transmitted during sexual intercourse. Among the male partners of infected women a high rate of infection has been found in prostate and urethra (47). This warrants treatment of both partners. The incidence of *Trichomonas vaginalis* is more among married and sexually active women than in postmenopausal and virgin females (79). These factors confirm that the infection

is transmitted sexually. In rare situations non-sexual modes of transmission via douche nozzles, specula or toilet seats have been reported (113). In 2-17% of female babies born to women infected with *T.vaginalis*, the protozoan parasite has been found in their urinary tract or vagina (5).

3.7 PATHOGENESIS

T. vaginalis is the most intensely studied trichomonad and is one of the world's common cause of non-viral STDs yet the exact mechanism of its pathogenesis have not been clearly understood. Many mechanisms are thought to be involved such as, cell-to-cell adhesion (8) and the excretion of soluble factors such as extracellular proteinases (82) and cell detaching factor (CDF). *T.vaginalis* produces cell detaching factor that enables the detachment of the cell a monolayer in vitro (27). The interaction of *T. vaginalis* with the resident flora of the vagina is thought be an important factor that leads to the development of the disease (69). The host- parasite relationship is very complex and it is understood that single pathogenic mechanism cannot be responsible for the variety of clinical symptoms. All clinical isolates of *T. vaginalis* appear to be capable of infection and disease production (82).

3.7.1 Adherence and Adhesins

Adhesion of trichomonads to the vaginal epithelial cells is essential in the pathogenesis of the parasite (6). This depends on time, temperature, and pH. There is a mosaic of receptors for adhesion of host extracellular matrix proteins which help in ligand receptor binding (14). The adhesion of the parasite to the epithelial cell is mediated by four adhesion proteins: AP65, AP51, AP33, and AP23 (10) which act in a specific receptor-ligand fashion (55). AP65 is encoded by

at least three genes in a multiple-gene family (6) which is similar to the genes encoding for malic enzyme (77). The adhesins are expressed on the surface with a highly immunogenic glycoprotein, P270 (8). Contact of the parasite with the epithelium leads to amoeboid transformation, production of pseudopodia and up-regulation of adhesin synthesis. Studies have shown that the side opposite to the undulating membrane and the recurrent flagellum of the parasite attaches itself to the epithelial cells (6). Laminin, localized in the basement membrane of the epithelium, promotes cell adhesion, differentiation, shape, and motility in normal cells, and also has chemotactic properties (84). But its role in pathogenesis of *T.vaginalis* is not clear.

3.7.2 Hemolysins

Since *T. vaginalis*, unable to synthesize lipids, erythrocytes may be the main source of fatty acids that are needed by the parasite. In addition to lipids, iron can be acquired by lysis of erythrocytes. For haemolysis to occur the presence of metabolically active parasite is necessary. Maximum hemolysis occurs in normal vaginal pH of 4.5. Trichomonad can attach itself to the erythrocyte via a specific ligand receptor interaction and on attachment there will be release of perforin-like proteins which form pores in the erythrocyte membrane. Finally, *T. vaginalis* detaches itself from the cell and cell lysis occurs. *T. vaginalis* has been observed to phagocytose erythrocytes. This property of hemolysis has been correlated to virulence of *T.vaginalis*.

3.7.3 Contact-Independent Mechanisms of Pathogenicity and Cell-Detaching Factor

It has been shown that a cell-free product of *T. vaginalis* contact dependent factor causes cytopathic effects in cell culture (27). Addition of cell-free filtrate of a *T. vaginalis* culture to a cell culture monolayer causes detachment and clumping the cells of the monolayer yet remain

viable. This is thought to be analogous to the sloughing of vaginal epithelial cells, seen in the vaginal mucosa during acute infections (27). CDF activity is probably a factor in pathogenesis. CDF, which is thought to be an extracellular factor and was found to be a 200-kDa glycoprotein which is heat and acid labile (6). Increasing production of CDF was associated with increased severity of clinical disease (27). CDF is also immunogenic. It is unclear whether the regulation of CDF production its activity, its immunogenicity play a role in the severity of symptoms. All these pathogenic mechanisms are probably important in the virulence of this disease.

3.7.4 Interaction with the vaginal flora

T. vaginalis thrives in pH of higher than 5 but the normal pH of the vagina is a very acidic (pH 4). The relationship between protective lactobacilli and *T. vaginalis* is not completely understood. It has been seen that with the rise in the vaginal pH there is either decrease or complete absence of *Lactobacillus acidophilus* and an increase in the number of anaerobic bacteria (69). It has been proposed that *T.vaginalis* may phagocytose *L. acidophilus* or the protienases and contact dependent factor secreted by *T.vaginalis* may destroy the bacteria (69).

3.7.5 Immune System Evasion

The ability to evade the host immune system is an important aspect of pathogenesis of *T. vaginalis.* This organism avoids complement to overcome the activity of human immune system. It has long been known that *T. vaginalis* activates the alternative pathway of complement (30). Cervical mucus is deficient in complement and the only source is the menstrual blood which has less of complement. Menstrual blood has appreciable complement-mediated cytotoxicity towards *T. vaginalis.* It was found that iron was a contributing factor in complement

resistance (9) has found that two classes of markers are alternately expressed on the surface of the organism: the highly immunogenic glycoprotein (P270) and the adhesins (AP65, AP51, AP33, and AP23) (77). The positive-phenotype (P270 positive) organisms lack adhesins and cannot cytoadhere or parasitize host cells (82). Only the organisms of the negative phenotype (P270 negative) which express adhesins have the ability to cytoadhere to the host cell (7). Adhesins of *T. vaginalis* has numerous other ways of evading the immune system. This parasite also secretes highly immunogenic soluble antigens which may neutralize antibody or cytotoxic T lymphocytes thus short-circuiting specific anti-*T.vaginalis* defense mechanisms. *T. vaginalis* can coat itself with host plasma proteins and does not allow the hosts immune system to recognize the parasite as foreign. Thus immune system mechanisms, such as presentation and antigen complement-mediated lysis do not occur (79).

3.8 CLINICAL MANIFESTATIONS:

Trichomoniasis presents with a number of different clinical patterns. The spectrum of clinical features may vary from asymptomatic carrier state to flagrant vaginitis. Within six months one third of asymptomatic women become symptomatic (85). Trichomoniasis is the disease of reproductive age group hence the clinical features are seldom observed before menarche or after menopause. *T.vaginalis* mainly infects the squamous epithelium of the genital tract and the infection in females lasts longer. The incubation period on an average is about 4 to 28 days.

Acute infection: Women with acute infection will have vaginal discharge which is frothy, green or yellow and muco-purulent associated with leucorrhoea (85). Edema and erythema is observed in 22-37%, along with pruritis and irritation. The discharge may be malodorous with pH > 4.5 (102). Small punctuate hemorrhagic spots appear in the mucosa of vulva and vagina which are

called colpitis macularis or strawberry cervix (91). The urethra may be infected in most women and they may present with lower abdominal pain and dysuria (91).

Chronic infection: These women mostly present with mild vaginal discharge along with dysparunia and pruritis. Vaginal discharges in them are not classical. These groups of women are the major source of transmission of the parasite. There are several complications reported in women following trichomoniasis.

a. Endometritis – *T.vaginalis* is thought to play a role in pelvic inflammatory disease. Cherpes *et al* (2006) investigated a relationship between endometritis and fallopian tube obstruction associated with *T.vaginalis* on 736 women by carrying out endometrial biopsy at enrollment and hysterosalpingography after 12 weeks. Women with infection with *T.vaginalis* had acute endometritis and more when associated with HSV-2 infection.

b. Premature rupture of membranes: pregnant women who had *T.vaginalis* in their vagina more often have premature rupture of membranes. *T.vaginalis* carriers are 1.4 times more likely to develop premature rupture of membranes (70).

c. Low birth weight- cervical infection with *T.vaginalis* has been shown to be associated with decreased gestational age at delivery. It has also been found that 50% cases of low birth weight are associated with asymptomatic trichomoniasis (34).

d. Infertility- *T.vaginalis* has been suggested as the cause of infertility in males and females. Benchimol *et al* (2008) has demonstrated the effect of *T.vaginalis* on human sperms and it revealed that when the sperm cells were exposed to *T.vaginalis* over different period of time, the trichomonads first adhere to the surface of the sperm cells, render them non-motile and then

gradually phagocytose and digest them with the help of Lysozymes. This is being thought as a mechanism of reproductive failure. A study done at Infertility Service of the National Institute of Endocrinology from June 1999 to June 2000, showed that out of 172 couples who visited this clinic for the first time, 10.5%were positive for the parasite where the women presented with leucorrhoea (87).

e. Cervical erosion – Reproductive tract infections have been shown to be associated with inflammatory changes in the cervical epithelium and has shown that there is an association between reproductive tract infections and cervical epithelial changes. Among the studied women it was found that 80.5% had inflammatory epithelial changes and of these 18.3% were detected to have *T.vaginalis* infection (96).

3.8.1 Clinical features in Men

Men are generally considered as asymptomatic carriers of *T.vaginalis* and these can be identified by investigating sexual contacts with infected women. Symptomatic men present with profuse purulent urethritis, while symptoms may simulate non-gonococcal urethritis (48). Complications in men infected with *T.vaginalis* include, prostatitis, balanloposthitis and epididmytis.

3.9 TRICHOMONIASIS AND HIV

Trichomoniasis is associated with HIV transmission. Presence of other STI's makes the patient susceptible to acquire and transmit HIV by two to five folds (100). All over the world, 75% of human immunodeficiency virus type 1 (HIV1) are acquired through sexual contact (111).

Since 1986 when HIV was first identified in India it has been spreading as an epidemic throughout the country. As per the latest estimate the number of HIV infected individuals varies between 2-3 million with prevalence of 0.36% (75).

Majority of the transmission i.e., about 81% of infection is transmitted through sexual contact, 6% through blood and blood products, 5% through intravenous drug use and less than 1% from mother to child (106).

In a study conducted in Eastern Zimbabwe, to look into the epidemiology of *T.vaginalis* infection and its association with HIV, using serological assays, 40.3% women were found to be positive for *T.vaginalis* and HIV (66). The HIV seropositvity was significantly associated with being sexually active; having multiple sex partners, having a partner who had multiple sex partners, seropositvity was associated with a recent history of genital discharge (66).

In a cross-sectional study conducted among 1,209 female sex workers in Ivory cost by bivariate analysis an association between HIV and Trichomonas infection showed a crude odds ratio 1.8, with 95% CI. In another cross-sectional study conducted in Tanzania among 359 women admitted to a hospital for gynecological conditions, Trichomonas was more common in women with HIV infection. Epidemiological studies have shown that ulcerative and non ulcerative sexually transmitted pathogens *N.gonorrhoeae, T.vaginalis* respectively are associated with increased risk of HIV-1 infection. Vigorous host immune response following physical trauma and damage from other sexually transmitted pathogens (STPs), compromise the integrity of mucosal surfaces thus potentially enhancing susceptibility to infection (33). As a result of local inflammatory response there is an infiltration of leukocytes, including CD4+ bearing lymphocytes and macrophages to which HIV can bind and gain access (56). In HIV infected

person the factors mentioned above can increase the level of virus- laden body fluids and the number of HIV virus infected lymphocytes and macrophages in the genital area thereby increasing the probability of HIV exposure and transmission to the uninfected partner. Cervical inflammation has been found to be associated with increased cervical shedding of HIV (46).

Increased urethral viral loads have been documented in men with Trichomonas infection (38).

T. vaginalis is able to degrade secretory leukocyte protease inhibitor which is known to block HIV cell attachment; this phenomenon may also contribute to HIV transmission (23).

In a study done among the HIV seropositive and HIV seronegative women the prevalence of trichomoniasis was 9.4%-29.5% and 8.2%-23.4% respectively **(**19**).** This was a HIV epidemiology study done among 871 HIV- seropositive women and 439 HIV- seronegative women from four sites in USA. These women were followed for 5.5years.trichomoniasis was diagnosed on the basis of wet mount and culture on modified Diamond's media. Like trichomoniasis, HIV infection is associated with low birth weight, premature delivery and 8-40% HIV transmission to neonates (110). Increased shedding of HIV in the semen with Trichomonas in urethera has been reported by Choen *et.a*l [2002]. *T.vaginalis* is associated with increased human immunodeficiency virus (HIV) shedding in women infected with both *T.vaginalis* and HIV (50).

3.10 DIAGNOSIS

(i) Wet preparation:

Diagnosis of trichomoniasis has traditionally depended on the demonstration of motile protozoa in vaginal and cervical secretions. This procedure was first described by Donne in 1836

Sensitivity is highly dependent on the expertise of the microscopist performing the test (62) Delay in transport processing of the sample in the laboratory may cause the organism to lyse or become non-motile (62). This method is inexpensive and can be performed rapidly (102). The minimum number of trichomonads required to appreciate the motility is 10^4(93).

(ii) Culture:

The "gold standard" test for the diagnosis of trichomoniasis has been culture in microaerophilic conditions (62). Several different media are available for culture. Most of the culture systems used is broth cultures (102). To initiate growth in culture the inoculums must contain at least 300-500 trichomonad/ml (98). The interpretation of broth cultures is simple.

a) Complex media

This was first described by Kuldan, Honiberg, Frost and Hollander in 1970. This media was for routine propagation of organisms and also to provide inoculation for a more defined media. This media was supplemented with 10% horse serum (57).

b) Tissue culture media-CMRL 1066

This is a modified tissue culture media. The fact that *T.vaginalis* is intimately attached to host cells and is able to multiply in co- cultures with suitable cells. Based on this modified tissue culture media were developed (57): the CMRL 1066 (Parker, LaRoy & McCulloch, in 1957) and NCTC 135 (Evans, Bryant, Kerr& Shilling).

The doubling time of trichomonas in this media was fifteen hours as compared to complex media which is ten hours. Sub-cultured organisms can be cryo-preserved in liquid media (57). CMRL 1640 is a modified tissue culture media prepared to supply cholesterol. This media contains commonly occurring fatty acids and bovine serum albumin to act as a stabilizer and supply all the other liquid factors. The doubling time of Trichomonas in this media is 14 hours.

c) Defined media

In this kind of media concentrates of vitamin, amino acids and trace elements are added. Single strength media can be stored frozen at -20˚c for 3 months or at 4˚C up to two weeks. Concentrates in this media can be stored at -20˚C up to 6 months (57).

d) DL-7 and DL-8

This is a very rich medium containing wide range of vitamins supplied by vitamin concentrates. It also contains purines, pyrimidines and amino acids. Ascorbic acid and cystine in the media reduce the redox potential. *T.vaginalis* grows rapidly in this media supplemented with 8% FCS. The doubling time of trichomonads is about 11 hours. Serum in this media was replaced by 'fatty acid poor' albumin and Armour 'fraction V 'albumin. The growth results were successful (57).

e) Kupferberg's STS Media Modified:

This media was discovered in 1955 by Kupferberg and was called Kupferberg's simplified "trypticase"serum (STS) medium. This medium was slightly modified later to help in the preparation of the media and to bring down the cost. The media constitutes of Maltose 1gm, Oxoid agar 2gm, trypticase (Baltimore Bilogical Laboratories) 20gm, cystine hydrochloride

1.5gm dissolved in 1 liter of distilled water. 0.2ml of 0.5% Methylene blue is used as an indicator. After autoclaving the media is supplemented with 0.25ml of sterile serum containing 100,000 units of penicillin and 20 mg of streptomycin. Advantage-S.T.S. media is a good transport medium and the positive cultures survive from 14-23 days (120).

f) Diamond's media modified: Klass modification

This is a commercial media. The Remel Diamond's media modified contains 12% horse serum (28). In a study done among 163 female patients attending Obstetric and Gynecology in Sinai Samaritan Medical centre, 32% patients were detected positive by modified Diamond's media and 24.5% by Kupferberg medium (28). Between the two commercially available medium used for diagnosis of *T.vaginalis,* Diamond's media modified proved to be superior (28). All the above mentioned culture media are liquid broth cultures.

g) Modified Columbia Agar (MCA)

This is a solid medium used for cultivation of *Trichomonas vaginalis*. This media contains Columbia Agar which is dissolved in distilled water, autoclaved for 5 min. After cooling sterile, inactivated, Lamb serum along with malt extract and dextrose are added. This is also supplemented with Baneocin, Nystatin, Penicillin, Streptomycin and Chloramphenicol. About 10ml of the mixture is poured into 6mm petri dishes. When kept at 4˚C media can be stored for a week (100). In a study done in Austria in patients attending out-patient clinic, MCA showed a sensitivity of 98.5% to detect *trichomonas vaginalis*.

h) In Pouch TV culture system

The In pouch culture system (Biomed diagnostics, San Jose California) is a commercially available system which is used for detection of *Trichomonas vaginalis.* In this system both the wet preparation and the culture systems are combined together. The System contains two pouches. Two swabs are inserted from an opening on the top. The upper pouch is used for culture while lower pouch is for wet preparation (93, 98). In a study done among 601 women among women attending a primary health clinic in Ballabgrah, Haryana, 22 women were positive by wet mount and 40 by In Pouch culture which was statistically significant with a P <0.001 (93). Advantages of this media are, it is stable for 6 months at room temperatures, specimen transport and culture can be done in the same media. The specimen can even be mailed and remain viable for a week (15). The sensitivity of In Pouch TV compared to Diamond's modified media is 97% (12).

iii) Papanicolaou Test

Papanicolaou staining has been used for screening genital infections and abnormalities. The main advantage of this method is that the samples can be stored permanently and can be reviewed later. Studies have shown that the sensitivity of this test ranges from 24%-67%. In a study done in Brazil, the specificity of Papanicolaou test was 97.6%, whereas sensitivity was only 60.7%. The positive predictive value of the Papanicolaou smear was 61.7% (58). There may be errors in diagnosis due to false positive and false negatives.

The main purpose for staining trichomonads is to optimize the visualization of key anatomic structures for accurate identification of the organism (63). Smears can be made from the vaginal discharge or cultures, after fixation stained with Giemsa stain, Silver stain, iron-hematoxyline and Acridine Orange (63).

iv) Gram stain

G. Ewart Cree in 1964, first observed vaginal smears by doing Gram's stain, and could visualize trichomonads, the nucleus, flagellae and axostyle. The method used to prepare the smears, is to dip a glass slide in the vaginal exudates in the posterior fornix and roll it over the exudate. Trichomonads appear gram negative and identified by lenticular eccentric nucleus and foamy cytoplasm (18).

v) Xenostrip

Xenostrip-Tv (Xenotope Diagnostics, Inc., San Antonio, Tex.) is an immunochromatographic strip test. It is a qualitative assay used to detect *T.vaginalis* –specific antigen by colour immunochromatographic "dipstick" technology. It is a rapid test as the results are ready within 10-20 minutes (50). In this assay mouse antibodies are bound to nitrocellulose membrane. The sensitivity and specificity of this test are 66.7% and 100% respectively (81). The Xenostrip rapid assay is useful in settings where prevalence of infection with *T.vaginalis* is moderate and microscopy is not possible (50).

vi) Latex agglutination tests

This test can be done on vaginal swabs. Kalon TV latex agglutination test (Kalon Biological, Surrey, UK) is used. Vaginal swabs are eluded in phosphate buffered saline by agitation. Mix one drop of this elude with one drop of latex on a black glass slide. Slide is rocked for 2 minutes. Agglutination indicates presence of *T.vaginalis* antigen(3). In a study done by Y Adu Sarkodie *et*

al among 3807 consecutive women in antenatal clinic at Ghana to compare wet mount culture and latex agglutination showed the sensitivity and specificity very much comparable to culture and far superior to wet mount (3).

vii) Molecular methods

Molecular probe for identification of *Trichomonas vaginalis* DNA. In a study done by Salvatore et.al a 2.3Kb DNA fragment of *Trichomonas vaginalis* was present in strains from diverse geographical regions were cloned (88) and DNA in vaginal discharge was detected using dot and blot hybridization technique. The probes used were Pros21, and Ppat22. These were radioactively labeled using random priming kits. Autoradiographs were prepared with Kodak diagnostic films. Disadvantages of this technique are the probe has some drawbacks like, radiolysis makes the probe instable, autoradiographic procedures are time consuming and preparation of clinical samples should correct handling and disposal of radioactive material (72).

Affirm VP Microbial identification test is a semi-automated commercial system (MicroProbe Corporation, Bothell, Wash) used for diagnosis of bacterial vaginosis and Trichomoniasis. In this assay synthetic oligonucleotide probes are used to detect *G.vaginalis* and *T.vagnalis* can simultaneously. Compared to culture and PCR the Sensitivity and specificity were 83% and 100% respectively (64).

Another molecular technique used is the fluorescent DNA In situ Hybridization. This is a technique in which the chromosomes of different organisms can be identified when a double stranded DNA is denatured and the single strand is exposed to a known nucleotide probe which

is labeled. This probe binds to the single strand and can be recognized by their fluorescence. In a study conducted by Ferris et al (1995), 501 symptomatic women were tested for *T.vaginalis* and candida using wet mount, pH and in situ hybridization. Trichomoniasis was detected in 7.4% by in situ hybridization method. The sensitivity and specificity was 86.5% and 98.5%, for *T. vaginalis* compared to culture.

Polymerase chain reaction (PCR) is a laboratory technique used for "amplifying" a specific DNA sequence. PCR is extremely sensitive and efficient and can make millions of copies of a specific DNA. Limitations of culture and microscopy led to the development of PCR in diagnosis of *Trichomonas vaginalis* infection.

Various studies have been done using different primers with different sensitivities and specificities (54). The sensitivity and specificity with these primers using vaginal swabs are varied sensitivity being 80 to 100% and sensitivity 88 to 100%. PCR has been performed on different types of clinical samples most commonly vaginal swabs and urine. PCR requires DNA of either viable or non viable organisms in very low concentrations (102). PCR of vaginal swabs may be useful in places where culture is not feasible in settings where self collection is utilized.

There are a number of primer sets that have been used as discussed above and these can be used to detect different genes or sequences of *T.vaginalis* each having a different product size. Primer set TVA5/TVA6 detects A_6P sequence of adhesin gene with a product size of 102bp, BTUB9/2 to detect beta tubulin gene with a product size of 112bp, TR5/TR6 detects $T_9$9 sequence with an expected product size of 154 bp, TR7/TR8 target T17 rp with a product size of 146 bp, TVK3/7 detects 312bp sequence from repetitive DNA and AP65 1/2 detects adhesin protein 65 encoding 65 KDa protein with a product size of 209 bp.

Colorimetric One –Tube Nested PCR:

This nested PCR was developed by Men-Fang Shaio et al (1997) from Taiwan. In this PCR, 650bp specific DNA repeats (Tv- E650) from *T.vaginalis* genome were targeted for amplification. The final product size amplified was 290bp. Four primers were used, from conserved region of *T.vaginalis* genome. They are outer primers1&2 (OP1/OP2) and inner primers 1and 2(IP1/2). The labeled inner primer sequence incorporated in the PCR product was captured in an avidin –coated microtiter plate and detected by antidigoxin alkaline phosphatase conjugate (94). Shaio *et al*. (1997) evaluated this nested PCR method by testing 378 vaginal discharge from symptomatic women. PCR and culture was positive for 31 samples. Among the 113 asymptomatic women tested 9 were positive by PCR, 2 of these were positive by wet mount and culture (94).

Recently a TaqMan –based real-time polymerase chain reaction also developed for the diagnosis of trichomoniasis. A study in which vaginal swabs taken from 119 women were tested by TaqMan based real time PCR and compared with wet mount and conventional PCR. This study showed that conventional PCR done on vaginal washings and urine had a sensitivity of 100% and 56.4% respectively while that for real time PCR for same samples were 100% and 76.7%. The specificity of both these tests were100% & 97.6% for vaginal washings and 82.9% & 97% for urine respectively (80).

3.11 TREATMENT OF TRICHOMONIASIS

Infection caused by *Trichomonas vaginalis* has been treated using topical vaginal preparations. These preparations give symptomatic relief but are unable to cure the disease (60). Local preparations do not penetrate the vaginal epithelium, urethra, Skene's gland and Bartholin's

gland where the infection is usually found (60) some local preparations include, clotrimazole, providone- iodine, nanoxynol 9 and some arsenical pessaries (60). There are no topical treatments for men (60).

After the discovery of Metronidazole in1959, it has remained the drug of choice for the treatment of Trichomonas vaginalis (97). Metronidazole (alpha–hydroxyethyl-2-methyl-5-nitroimidazole) is a 5-nitroimidazole, a heterocyclic compound with a nitro group on the fifth position of imidazole ring. It is a derivative of Streptomyces antibiotic azomycin (73).

Metronidazole is a prodrug and needs to be activated in susceptible organisms (73). The drug enters the cell membrane of *T.vaginalis* by passive diffusion and is activated by its reduction nitro group (cytotoxic nitro radical anion) (73). Transient binding of this nitro group to DNA causes, disruption and breaking up of strands leading to cell death (63). In *E.coli,* this DNA damage due to direct binding of the drug can be repaired by excision pathway which is not activated in *T.vaginalis* (89). Studies have shown that action of metronidazole is associated with thymine and thymidine phosphates (79). Since the genome of *T.vaginalis* has about 71% of A+Tcontent this parasite is attracted to metronidazole (89). The organanelle contains electron transport components linked to private: ferroxidine oxidoreductase (PFOR) which is responsible for activation of metronidazole (89).

When taken orally or intravenously, the bioavailability of the drug is 93-100% (116) which vaginal absorption of the drug in vagina is low with a bioavailability of 20% (89). Absorption of local preparations of metronidazole in the vagina are affected by, drug formulation (insert or cream), dose and physicochemical nature of the vagina at the time of treatment (89).

3.11.1 Treatment Regimens

Centers for disease control and prevention guidelines recommend 250mg three times a day for 7 days, 500mg twice a day for 7 days or a single dose of 2-g dose. Considering the patient compliance a 2-g oral dose has been considered adequate for the cure of *Trichomonas vaginalis* infection (59). Though unlike the treatment duration of 7 days, this regimen does not protect against re-infection unless both sexual partners are treated simultaneously. Cure rates of oral and intravenous regimens is 85 to 95% (59).

3.11.2 Treatment of pregnant women and lactating women- A single dose of metronidazole 2gm orally is the current treatment recommended by CDC in all stages of pregnancy. Daily intravaginal dose of 100mg clotrimazole for 6 days can be used for temporary relief during first trimester followed by metronidazole in second trimester has also been recommended (89).

A single dose of 2-g orally followed by a 24 hour interruption in breast feeding is recommended, thus preventing the exposure of the neonate to the drug (89).

Neonates-Neonatal trichomoniasis depends on the maternal estrogen level, having a spontaneous resolution as the level of estrogen comes down by third to sixth week. In children with persisting infection for more than six weeks or in presence of symptoms, the infant must be treated with metronidazole as single dose 50 mg/Kg or 10-30 mg/Kg daily for 5-8 days (89).

3.11.3 Metronidazole resistance:

Trichomonas vaginalis develop resistance to metronidazole either aerobically by oxygen scavenging pathways (79), or ferroxidine pathway (109) and aerobically by reduction or cessation of PFOR and hydrogenase (89). At least 5% of infections caused by *T. vaginalis* are due to strains which low or moderate resistance to metronidazole (74). After ruling out all the

causes of treatment failure a possibility of resistant *T.vaginalis* infection must be considered. Metronidazole susceptibility test must be done in all suspected cases. Relapse of infection in patients in whom non-compliance and re-infection should be ruled out. Factors which are responsible for relapse are, low zinc concentration in serum (115), inactivation of metronidazole by vaginal bacteria and ineffective delivery of drug to the vagina. These patients can be treated using the following protocol (4).

There are reports of refractory case as well. A refractory case is defined as cases in which two standard courses of treatment fail to cure the infection. To treat these cases, doses of oral metronidazole can be increased along with the extension of the course, which has been found to be effective in 80% of patients (74). These patients can be treated according to protocol described above (4). The treatment can be combined with vaginal clotimazole, arsenical pessaries, betadine douches and Solco Trichovac Lactobacillus vaccine (89).

Tinidazole

Tinidazole a 5- Nitroimidazoles compound is also used for the treatment of trichomoniasis. The half life of this drug is longer than metronidazole (68). The concentration of Tinidazole in the vaginal secretions is equal to that in the serum. Since the cross resistance between Nitroimidazoles is incomplete (74), Metronidazole resistance trichomoniasis can be treated with Tinidazole. In a study done at CDC on metronidazole susceptible and resistant *T.vaginalis* isolates, Tinidazole was effective against all metronidazole susceptible strains but killed only 3 of 12 metronidazole resistant strain (74). While Furazolidone was very effective for both strains of *T.vaginalis* in 2 to 3 hrs of exposure (74).

Other Nitroimidazoles compounds like Orinidazole and Secnidazole have a longer half life and lower rates of elimination. In a study among 20 women with trichomoniasis infection, a single dose of 2g was able to cure all patients (97). The other treatment option is the Nonimidazole chemotherapeutic agents, Hamycin. This is an aromatic polyene related to amphotericin B. This drug binds to ergosterols in the plasmalemma, which helps in formation of pores. There is leakage of the cytoplasm which leads to cell death. Hamycin in low concentrations kills both metronidazole sensitive and resistant strains of *T. vaginalis*. This drug is being used in India as topical treatment of trichomoniasis (117).

3.11.4 Vaccines

The rise in the number of refractory cases, and lack of alternative treatment, disease prevention with vaccine is considered. *T.vaginalis* does not induce long term immune protection (89). Thus the vaccine did not show any direct immune or antibody response to *T.vaginalis*. The vaccine stimulated an increase in non specific immune factors relieved the symptoms and removed infection. A *Lactobacillus* vaccine called Solo Trichovac was introduced by a Swiss company, Solco (Basel). This is a systemic vaccine containing eight aberrant strains of *Lactobacillus acidophilus* which had been isolated from *T.vaginalis* infected persons. The efficacy of this vaccine is 90-100% (32)

MATERIAL AND METHODS

4.0 MATERIAL AND METHODS

The study protocol was approved by the institutional review board. The study had two parts. The first part of the study was to standardize a non-nested PCR for the amplification of the one of the *Trichomonas vaginalis* gene and to evaluate its performance on frozen culture positive and culture negative samples. The samples for these culture supernatants were collected from women, who had presented with vaginal discharge. These women belonged to a rural area looked after by the community department of Christian Medical College. The vaginal swabs from these women were collected between the year 2005- 2006 and inoculated into modified Diamond's media.

After checking the media for growth for 10 days, the culture supernatants (2ml) were aliqouted in screw capped vials and stored at -20˚c. A total of 30 culture positive samples and 40 culture negative samples were included for the evaluation of the PCR.

For standardization of polymerase chain reaction culture concentrates and culture supernatants of two stored aliquots, one each of positive and negative culture of *Trichomonas vaginalis* were used.

In order to prevent PCR amplification product carry-over contamination, DNA extraction, master mix reagent preparation and handling of post amplification products were carried out in separate rooms designated for each of the above mentioned purposes. Each room had dedicated pipettes and tips with aerosolized barrier.

4.1 DNA Extraction*:*

DNA from *T.vaginalis* culture was extracted using CHELEX method (62). One and a half ml of stored culture supernatant was centrifuged in a micro centrifuge at 1000 rpm for 1 minute. Subsequently the supernatant and concentrates were separated out. For standardization, DNA was extracted from undiluted culture supernatants, undiluted and 1 in 10 dilutions of the concentrates. Fifty micro litter of the culture supernatant (neat sample), and 50µl of the concentrate was transferred to two 1.5ml tubes. The 1/10 diluted concentrate was prepared by the addition of 50µl of the concentrate to 450µl of sterile water. Fifty micro litter of this diluted concentrate was also taken in a 1.5 ml tube. Subsequently 200µl of 5% suspension of chelating resin (chelex 100, Sigma Aldrich, Steinhein, USA) in Tris buffer (0.01MpH 8.0) was added to these three tubes. This mixture was incubated at 56˚C in a water bath for 30 minutes, followed by gentle vortexing. Subsequently this mixture was boiled in a water bath at 100˚C for 10 minutes and then centrifuged in a micro centrifuge at 1200g for 1 min. The extracted DNA was stored at -70˚c in multiple aliquots (62). Just prior to the PCR an aliquot was taken centrifuged in a micro centrifuge at 1200g for 1 min. The supernatant was discarded and the pellet was reconstituted in about 50µl of the remaining supernatant in the tube. The required DNA input was taken from this tube.

4.2 Polymerase Chain Reaction (PCR):

For standardization of the non nested PCR two sets of primers were tried. The first set using the TVA5/TVA6 which amplifies a 102 bp genomic sequence of adhesine gene (62). The second set of primer used was BTUB9/2, which amplify 112bp genomic sequence of beta tubulin gene. The sequences of the two sets of primers used were shown in Table 4.1.

Table 1. Nucleotide sequences of the primers used for *T.vaginalis* amplification

Primer Name	Nucleotide Sequence	Region amplified	Product size	Reference
First set of PCR Primer 1: TVA5 Primer 2: TVA6	5'GATCATGTTCTATCTTTTCA3' 5'GATCACCACCTTAGTTTACA3'	A6p	102bp	(62)
2nd set of primer Primer 1: BTUB9 Primer 2: BTUB2	5'CATTGATAACGAAGCTCTTTACGAT3' 5'GCATGTTGTGCCGGACATAACCAT3'	btub1,2 and 3	112bp	(62)

The Master Mix was set up to a final reaction mixture volume of 40µl by adding sterile distilled water. For each reaction 10µlof DNA template was added. The concentration of the each reagents used for the preparation of master mix is shown in Table 4.2. After the addition of DNA the tubes were lightly vortexed and then centrifuged in a micro centrifuge and kept in thermal cycler. (AB Applied Bio systems, Gene Amp PCR Systems 9700,USA)

Table 2. Master Mix PCR reagents used per sample for the first and second primer sets for the PCR

Reagent	Volume	Final concentration
PCR Buffer	5µl	1x
$MgCl_2$	5µl	1.5mM
Primers	2µl each	25p moles each
DNTPs	1µl	0.1Mm each
Taq polymerase	2µl	3.5 units
DNA	10µl	5µl
Final Volume	50µl	

A touchdown method for thermal cycling was used. The thermal cycling condition is given in Table 4.3. Annealing temperature begins at 62˚C and ended at 52˚c for 45secs Annealing temperature was lowered by 1˚C for every 4 cycles until reaching 52˚c. The total time taken for the assay was approximately 5 ½ hours.

Table 3. Thermal Cycling Conditions used for the PCR:

	Temperature	Time	Cycles
1st PCR	95°C	75sec	1
	95°C 62 °C-52 ° C* 72°C	45sec 45sec 45sec	60
	72 ° C	7 min	1

***** Annealing temperature was lowered by 1˚c for every 4 cycles until reaching 52˚c.

	Temperature	Time	Cycles
1st PCR	95°C	75sec	1
	95°C 62 °C-52 ° C* 72°C	45sec 45sec 45sec	60
	72 ° C	7 min	1

4.3 Detection of amplified targets:

The product of the PCR was checked for amplification by eletrophoresing in 2% agrose (Sigma Aldrich, Steinhine, USA) gel incorporated with 10µg/ml (0.01Mm)(62) of ethidium bromide. The electrophoresis was carried out in a submarine gel unit at 120 volts. Following the electrophoresis the gels were viewed in an UV transilluminator and documented using the gel documentation system (BIO-RAD, California, USA). The expected size of PCR product was 102bp for the adhesion gene and that for Beta tubulin gene was 112bp .The size of the amplified product was assessed by comparison with 50bp molecular marker (Fermentas Life Sciences, Canada, USA).

After standardization using the best performed primer set the test was evaluated with 30 positives and 40 negative culture supernatants. The statuses of the culture were blinded to the performer of the test. After completion of the testing of 70 culture proven samples, the numbers were decoded, and the results were compared with culture. For the analysis culture is considered as the "gold standard "for the diagnosis of trichomoniasis. On comparison the results were found quite unsatisfactory. It was decided to use a different PCR reagent i.e., HotStar Taq Master Mix (QUAGEN, Hielden, Germany) from another commercial source.

The reaction mixture was mixed well and then 5µl of DNA template was added to each tube. The final volume of the reaction mixture was 25µl. The mixture was well centrifuged in a micro centrifuge at 1000rpm /min for 1 minute, and the tubes were placed in the thermal cycler. The HotStar Taq Master Mix reagents used per sample for the PCR assay is shown in Table 4.4.

Table 4. HotStar Taq Master Mix PCR reagents used per sample

Component	Volume/reaction	Final Concentration
HotStar Taq Master Mix	12.5µl	2.5units HotStar Taq DNA Polymerase 1Xpcr Buffer 200µM of each Dntp
Primers	0.25 each	25pmol/reaction
RNase –Free water	7µl	-
Template DNA	5µl	-
Total volume	25µl	-

4.4 Prospective study in HIV infected women

The second part of the study was a prospective study to detect the prevalence of Trichomoniasis among HIV seropositive women. Vaginal swabs were collected from women attending infectious disease (ID) clinic of Christian Medical college Hospital and the hospital attached to the Community Medicine Department (CHAD). Though it was proposed to collect consecutive samples because of some logistic reasons there was a break in the sample collection and hence the sampling used was only a convenient sampling method. The study period was from the month of June 2006-Feburary 2007 and later again from Nov.2007-July 2008.

4.4.1 Sample size:

Sample size for the prevalence study of trichomoniasis among women with HIV seropositivity was calculated based on three Indian studies done by Mathai *et al* (1998) Divakar *et al* (2000)

and Sharma *et al* (1991) using a prevalence of 6% and desired precision of 3.5% with 95% C.I. The minimum number of samples to be tested was 177 (Epi info.ver.6.04).

4.4.2 Inclusion criteria

All HIV sero-positive women who attend the infectious disease clinics at the main hospital or the community hospital for the first time. The HIV seropositvity was confirmed by NACO testing strategy III using three tests.

4.4.3 Exclusion criteria:

Women whose HIV serology confirmed negative at our institution were excluded from the study. Two hundred and four women attending the infectious disease clinic for the first time were recruited in the study with a written informed consent. Vaginal discharge was collected by the physician using 3 cotton swabs stick from the posterior vaginal fornix under vision. One swab was used to prepare wet mount preparation to check for the motility of the parasite. Wet mount was checked in the laboratory attached with the ID clinic using a microscope at 10X and 40X objective respectively by the technicians in both ID clinic at the main hospital and community hospital (CHAD).

The second swab was inoculated into the culture broth (Diamond's media) while the third swab was inoculated into 2ml of sterile normal saline and stored at -20˚C for PCR.

The inoculated culture broth was incubated at 37^{o}C, and was checked for 10 days, by making wet preparation from broth to check for characteristic quivering motility of the trophozoites of

Trichomonas vaginalis every day. The swab sticks inoculated in the media were squeezed and discarded after 24 hours of incubation at 37˚C.

The third swab in 2ml saline was vortexed and discharge from the swab was washed in saline. After discarding the swab, the washed saline was aliqouted into 2 ml screw capped vials and was stored at -20^{o}C for PCR.

PCR was then preformed on vaginal discharge stored in saline. The sample was centrifuged and the DNA was extracted from the concentrate as mentioned in section 4.1 the protocol of PCR and the cycling conditions remained same as the one used for the evaluation of 70 culture proven samples with HotStar Taq DNA (Quaigen, Hielden, Germany). Negative water controls were used after every fifth samples. In order to avoid product carry over the PCR was set up in a separate room away from area involving amplified target sequence, thermo cycling areas, and PCR product storage and gel electrophoresis. Separate pipetting devices were used to set up PCRs and aerosolized barrier pipette tips were used.

Adjudication of discrepant samples (those found to be culture negative and PCR positive or PCR negative and culture positive) was done by repeating the PCR three times. A sample was considered to be *T. vaginalis* positive if, at least on two occasions PCR results were positive.

4.4.4 Preparation of Modified Diamond's Media:

Commercial Trichomonas base (Himedia-M1204) was used for preparing the media. This base contains the following ingredients:

Casein enzymic hydrolysate	17 gm/lt
Papiec digest of soybean meal	3 gm/lt
Liver digest	18 gm/lt
Glucose	2.5 gm/lt
Sodium Chloride	5.0 gm/lt
Di potassium hydrogen phosphate	2.5 gm/lt
Calcium Panthothenate	0.005 gm/lt
Chloramphenicol	0.125 gm/lt
Sterile sheep serum (inactivated)	10 ml/ 100ml
Streptomycin sulphate	0.1/ 100ml
PenicillinG (100,000U)	0.6 gm/100ml

Procedure:

The media was prepared by the addition of 8.17 gm of Trichomonas base to 90 ml of distilled water. Subsequently this mixture was heated till the media is completely dissolved. The pH of the media was adjusted to 6.2 ±0.2 followed by autoclaving the media at 121˚C at 15 Ib pressure for 15 minutes and cool it to 45˚C - 50˚C. This is followed by the addition of 10ml of heat inactivated sheep serum (inactivated at 56˚c for 30 minutes) and antibiotics (Penicillin G and Streptomycin sulphate) to the media and mixed well. Five milliliter each of this final media was dispensed in screw capped tubes and stored at 4˚C.

4.4.5 Quality checking of the media

Media was prepared in batches. Since ATTC Strains were not available, the quality of the media was checked by sub-culturing 2ml of the positive cultures in the previous batches into two tube of media prepared in the new batch and checking for motile trichomonads.

4.5 Statistical analysis:

The accuracy indices of the PCR and the wet preparation in comparison with the broth culture in Diamond's media were calculated by using the software Epi info.Ver6.04. The difference in the proportion of the positive samples in asymptomatic and symptomatic HIV infected women were analyzed by Ch2 test using the software Epi info.Ver6.04. The Kappa coefficient analysis to compare the agreement between the performance of culture and the non-nested PCR was carried out by the software Epi info.Ver6.04.

RESULTS

5 . RESULTS

5.1 Standardization of PCR

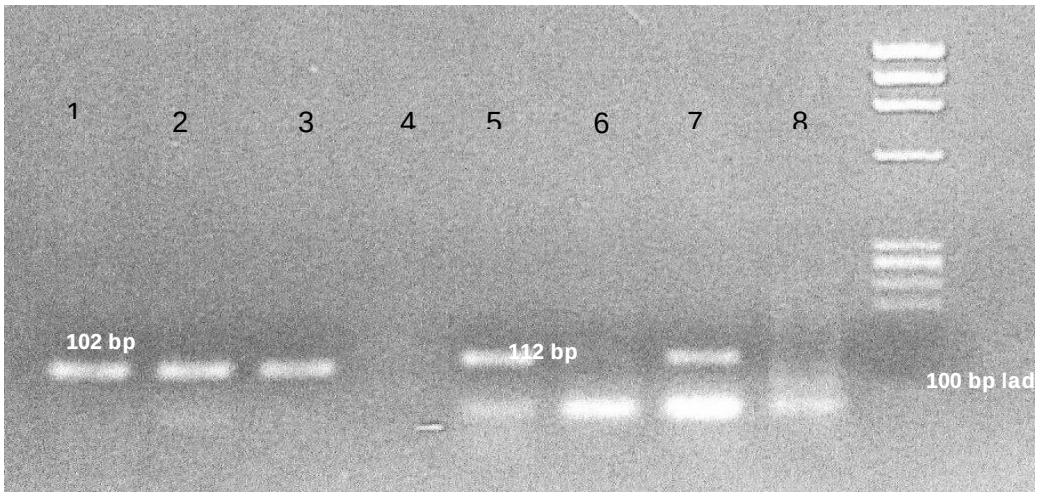
The first set of primers (TVA5/ TVA6) amplified the *Trichomonas vaginalis* DNA from all the three samples i.e., undiluted supernatant, undiluted concentrate and 1/10 diluted concentrate. The expected band size for the product was 102bp. The second set of primers, Beta tub9/2 also showed amplification with all the three samples with different dilutions used. However, the undiluted concentrate showed a very weak band. PCR was repeated with same sample and primer set Beta tub9/2 where two concentrations of primers were tried, 25mM/ reaction and 10mM/ reaction, the concentration of other reagents as well as cycling conditions remained same. There was no improvement in the results. The gel picture showing the *Trichomonas vaginalis* specific bands with different concentrations of samples with the two sets of primers used are shown in **Figure 1.**

Since the results with the TVA5/TVA6 primer set was better than the Beta tub9/2 primer set it was decided to continue the study with TVA5/TVA6 set of primers.

5.2 Evaluation of the PCR with Trichomonas vaginalis culture proven sample

PCR on 70 culture proven samples were done using the primer set TVA5/TVA6. Among the 30 culture positive samples only 13 (43.3%) were showed an expected band size (102bp) while among the 40 culture proven negative samples 39 were negative. The accuracy indices obtained for this assay when compared to the culture results is shown in **Table 1.**

Figure 1: The Gel picture showing the *Trichomonas vaginalis* specific bands with different concentration of culture proven samples with the two sets of primers used in the study



Lane 1 - 4 with primer set TVA5/ TVA6 (102bp) while lane 5-8 primer set beta tub 9/2(112bp)

Lane 1 neat sample (culture supernatant)

Lane 2 concentrated sample (deposit of the centrifuged culture supernatant)

Lane 3 diluted sample (1/10 dilution of the concentrate) and

Lane 4 negative control

Lane5 neat sample (culture supernatant)

Lane 6 concentrated sample (deposit of the centrifuged culture supernatant)

Lane7 diluted sample (1/10 dilution of the concentrate)

Lane 8 negative control and Lane 9 Molecular weight marker

Table 1: The accuracy indices of the *Trichomonas vaginalis* PCR assay using *Taq polymerase,* Nucleotides and buffer mixtures (Ferments life Sciences, Canada, USA)

		***T.vaginalis* Culture status**	
		POSITIVE	**NEGATIVE**
T.vaginalis **PCR assay**	**POSITIVE**	13	1
	NEGATIVE	17	39

SENSITIVITY	43.3% (26.0 –62.3) [#]
SPECIFICITY	97.5% (85.3 – 99.5)
POSTIVE PREDICTIVE VALUE	92.9% (64.2 – 99.6)
NEGATIVE PREDICTIVE VALUE	69.6% (55.7 – 80.8)

[#] Figures in parentheses are the 95 % confidence interval values

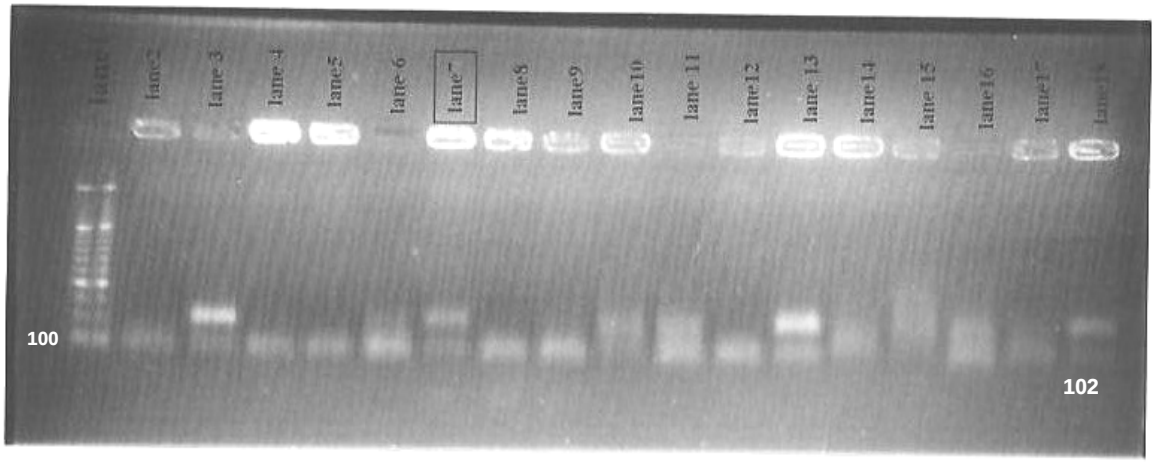
Figure 2 shows the gel picture showing the *Trichomonas vaginalis* specific bands with culture positive and culture negative samples with first set of PCR reagents (Ferments, Life Sciences, Canada, USA)

As the PCR assay showed less than 50 % sensitivity with reagents used, it is decided to use a hotStar Taq Master Mix from another commercial source (Qiagen, Hilden, Germany). Two positive cultures, ID No. HP162 and ID No. HP165 which had shown bands in the assay with the earlier reagents were used. A positive and a negative control were also included in the run. Both the 25µl and 50µl PCR reactions showed band with the two known positive samples and the controls were satisfactory. **Figure 3** shows gel picture with *Trichomonas vaginalis* adhesion gene specific bands in 50µl and 25µl reaction PCR assay done on culture positive samples.

Both the 25µl and 50µl PCR reactions showed bands with similar brightness for both the test samples and the controls. Subsequently all the 70 culture proven samples were tested with 25µl reaction mixture using the HotStarTaq master mix (Qiagen, Hilden, Germany) reagents. Among the 30 positive samples 29 were found to be positive for *Trichomonas vaginalis* adhesion gene product. Of the 40 culture negative samples all were negative except one. **Figure 4** shows gel picture of the PCR assay done on culture proven samples with HotStar Taq Master Mix.

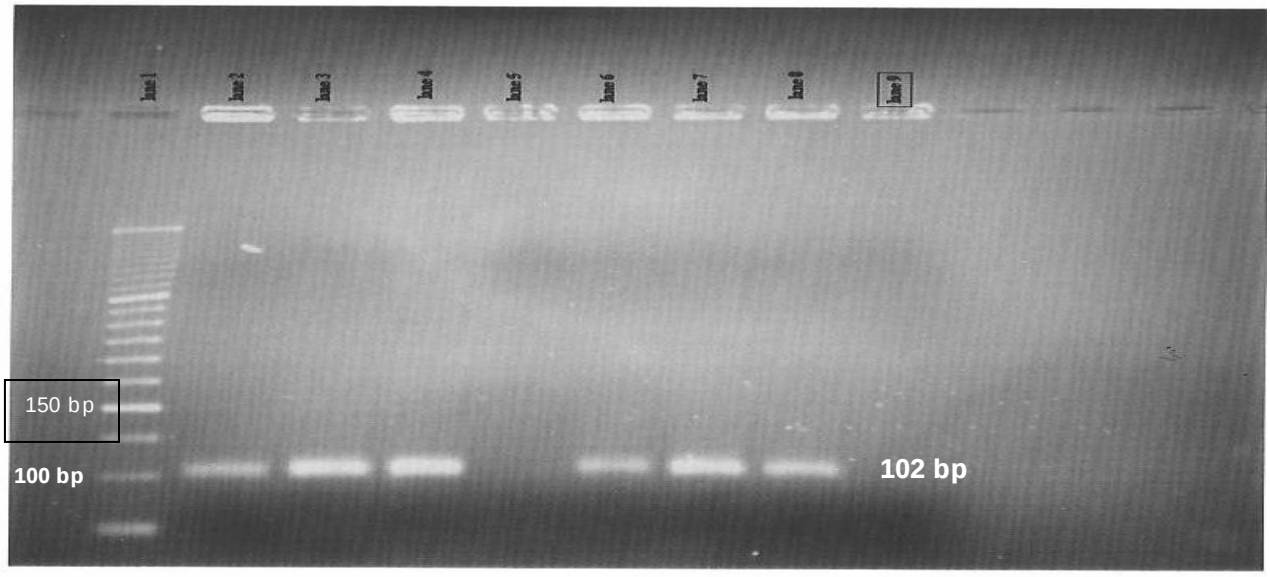
The accuracy indices of the *Trichomonas vaginalis* PCR assay with HotStarTaq master mix reagents (Qiagen, Hilden, Germany) compared to culture results are shown in **Table 2.**

Fig 2: The Gel picture showing the *Trichomonas vaginalis* specific bands with culture positive and culture negative samples (first set of PCR reagents).



Lane1	molecular weight marker
Lanes 6, 11, 17	negative water control
Lane18	positive control
Lanes 2, 3, 4, 5, 7, 8, 9, 10, 12, 13, 14, 15, 16	blinded cultures

Fig 3: The gel picture showing the *Trichomonas vaginalis* specific bands in the 50 µl and the 25 µl reaction PCR assay done on culture positive samples.



Lane 1 Molecular weight marker

Lanes 2 Id No. HP162

Lane 3 Id No. HP165

Lane 4 Positive control of culture proven samples in 50µl reaction.

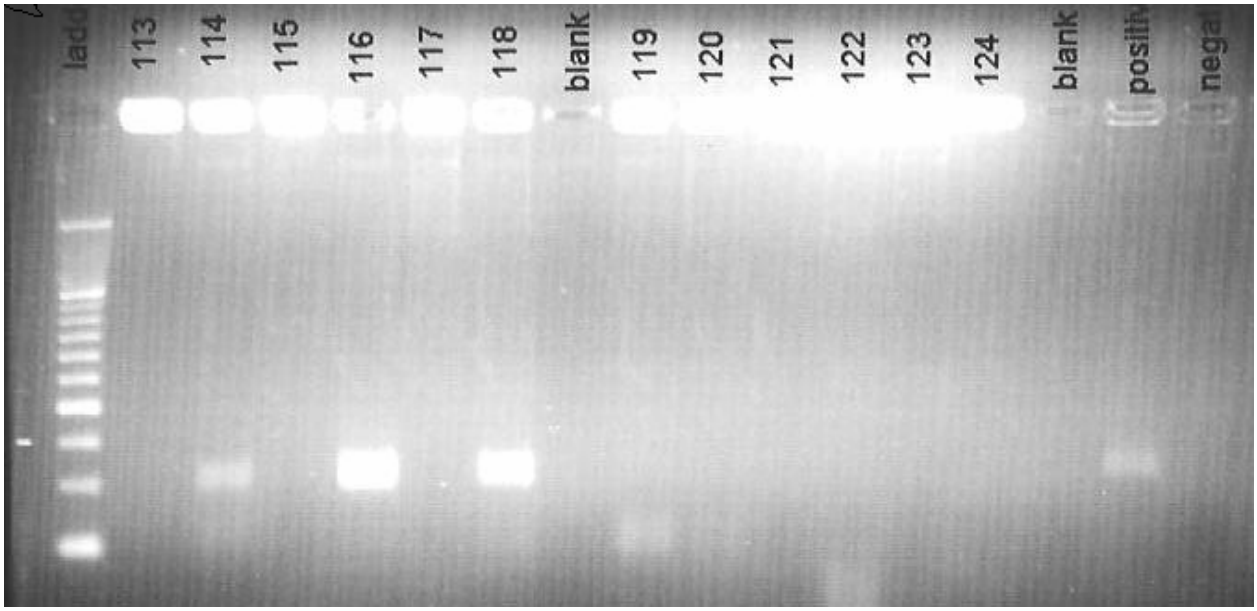
Lane 5 Negative control

Lanes 6 Id No. HP162

Lane 7 Id No. HP 165

Lane8 Positive control of culture proven samples in 25µl reaction.

Figure 4: Gel picture of PCR assay done on cultures proven samples with HotStar Taq *Master Mix.*



Lane1-molecular weight marker,

Lanes3,5,7, blinded culture samples with *Trichomonas vaginalis* specific bands

Lanes 8, 15 , - negative control water

Lane 16-Positive control

Table 2: The accuracy indices of the *Trichomonas vaginalis* PCR assay with HotStarTaq master mix reagents (Qiagen, Hilden, Germany)

		***T.vaginalis* Culture status**	
		POSITIVE	**NEGATIVE**
T.vaginalis **PCR assay**	**POSITIVE**	29*	1
	NEGATIVE	1	39

**two samples amplified only after testing with 1/10 diluted DNA*

SENSITIVITY	96.7%	(80.9. – 99.8) [#]
SPECIFICITY	97.5%	(85.3 – 99.9)
POSTIVE PREDICTIVE VALUE	96.7%	(80.9 – 99.8)
NEGATIVE PREDICTIVE VALUE	97.5%	(85.3. – 99.9)

[#] Figures in parentheses are the 95 % confidence interval values

5.3 Determination of the frequency of Trichomoniasis infection among the prospectively collected samples from HIV infected women attending infectious disease clinics

A total of 204 women were recruited in to the study. Among this 2 were found to be HIV negative on repeat testing. From 2 individuals, swabs which were to be collected for the PCR assay were not done. There was over spillage of sample from the tubes and hence discarded. Samples from these 6 individuals were excluded for the study analysis.

5.3.1 Source and Demography of study participants

Out of the 198 women 28 (14.1%) were recruited from the infectious disease (ID) clinic of the community hospital (CHAD) while 170 (85.8%) were taken from ID clinic of the main Christian Medical College, Hospital. This data is shown in **Table 3.**

Among 198 study recruits, majority of the patients belonged to the states from southern India 180 (91%) The region wise distribution of the study recruits is shown in **Figure 5**

All the four South Indian states and the one union territory had a representation among the 198 women included in the study. Majority of the patients belonged to Tamil Nadu 140 (77.7%), while 36 (20%) came from Andhra Pradesh. The state wise distribution of the study participants from south India is shown in **Table 4**

The mean age of the study participants were 33.28 (SD= 7.28 yrs, Range 21-52 yrs).

Table 3: Sources (ID clinic) of the prospective study participants (n=198)

Source	Number	Percentage %
ID Clinic CHAD	28	14.1
ID Clinic CMCH	170	85.8

Figure 5: Regional Distribution of Study Participants (n=198)

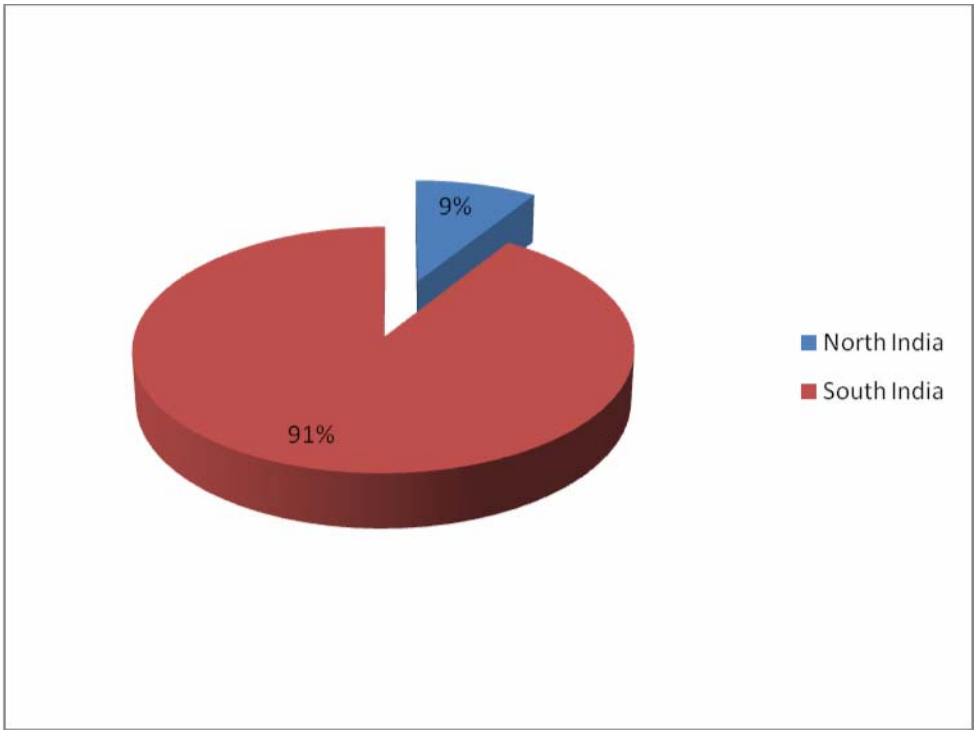


Table 4: The state wise distribution of the prospective study participants from south India (n=180)

States	No. of Patients	Percentage
Tamil Nadu	140	77.7
Andhra Pradesh	36	20.0
Karnataka	1	0.55
Kerala	1	0.55
Pondicherry	1	0.55

Table 5 shows the age wise distribution of 198 HIV seropositive women in the study. It shows that predominance of two age groups, women of 30-49 years of age which accounted for 98 (49.49%), followed by 20-29 years of age that constitutes 69 (34.84%) of the 198 cases. Among the 198 women majority 180 (91%) were house wives while the remaining 18 (9%) were working women. This data is shown in **Figure 6.**

Fifty five women among the study participants were symptomatic. Twenty seven among these 55 women presented with vaginal discharge while the remaining 28 were found to have discharge during per vaginal examination. **Figure 7** shows the distribution of the study participants based on their symptoms.

5.3.2 The *Trichomonas vaginalis* positivity among the prospectively collected samples from HIV infected women by three different assays

Among the 198 samples tested by wet mount only 1 (0.5%) were found to be positive for *Trichomonas vaginalis* trophozoite. **Figure 8** shows the representative wet preparation showing *Trichomonas vaginalis* trophozoite. A methylene blue stained preparation of the *Trichomonas vaginalis* trophozoite in a wet preparation from a culture positive HIV infected woman is shown in **Figure 9**

Six (3%) of the 198 samples showed positivity in modified Diamond's culture medium. Four of these samples showed positivity in the broth culture by 24 hours. The other two showed

Table 5: Distribution of the prospective study participant in the different age category (n=198)

Age in years	No. of HIV infected women	Percentage
20-29	69	34.84
30-39	98	49.49
40-49	22	11.11
50-59	9	4.54

Fig 6: The distribution of the study participants based on their occupation (n=198)

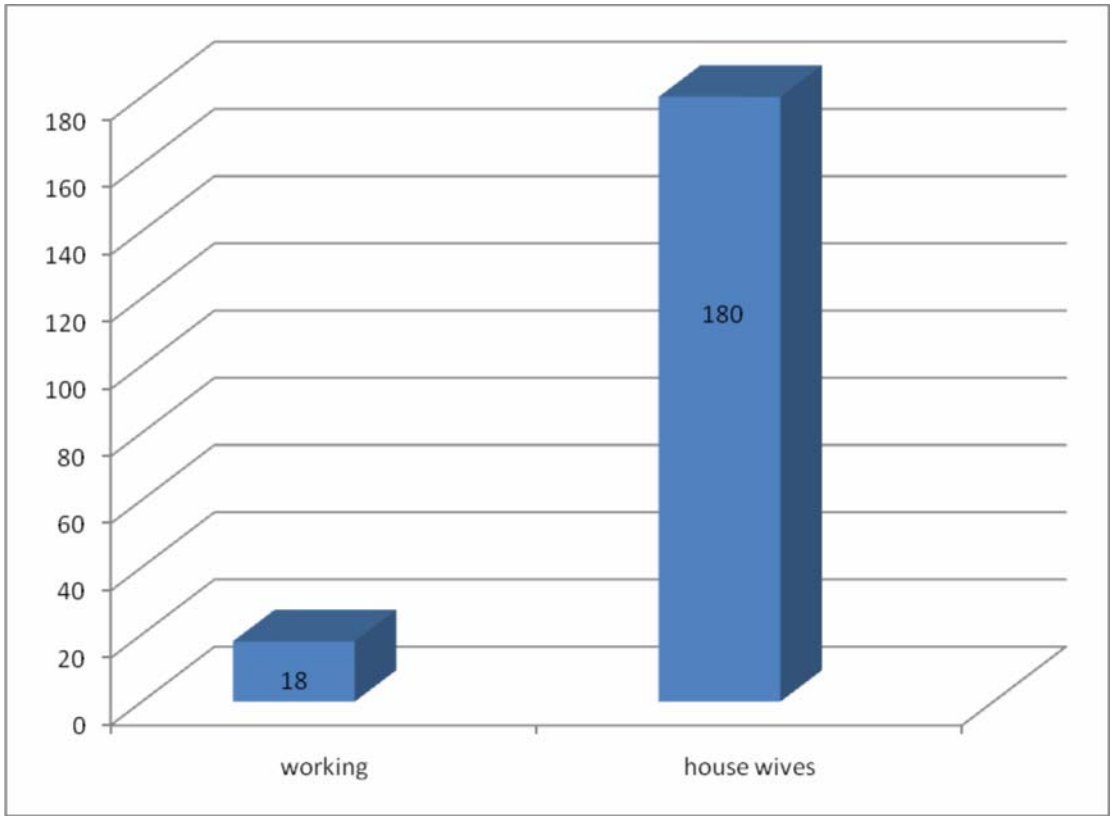


Fig 7: The distribution of the study participants based on their symptoms (n=198)

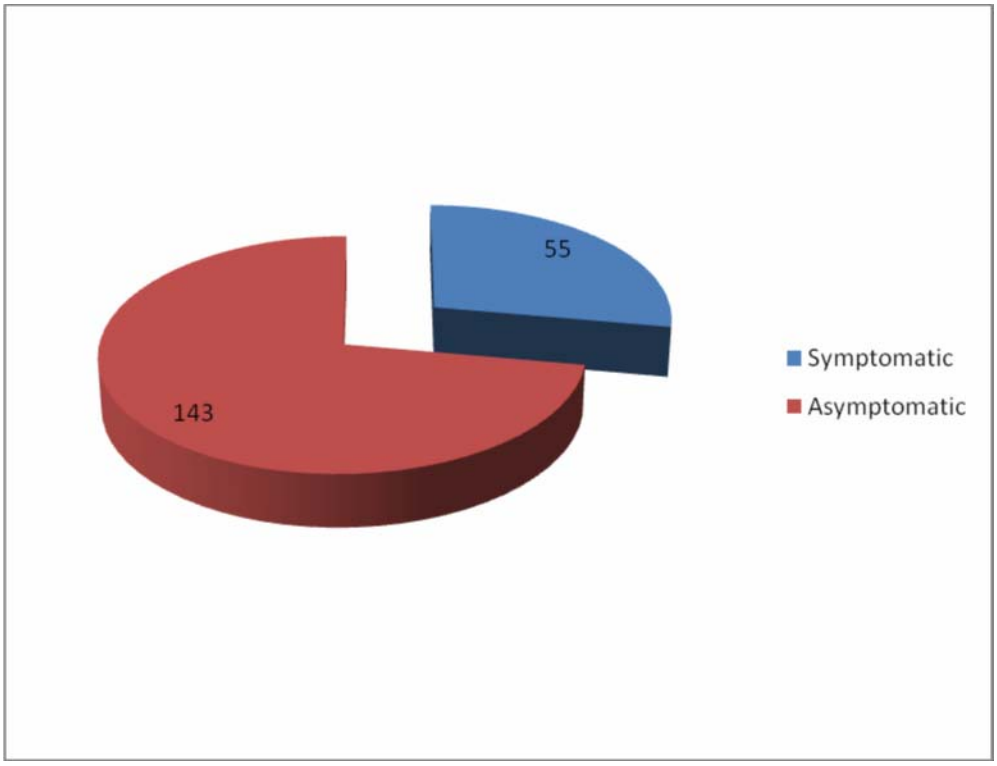


Fig 8: Trophozoites of *Trichomonas vaginalis* in wet mount preparation

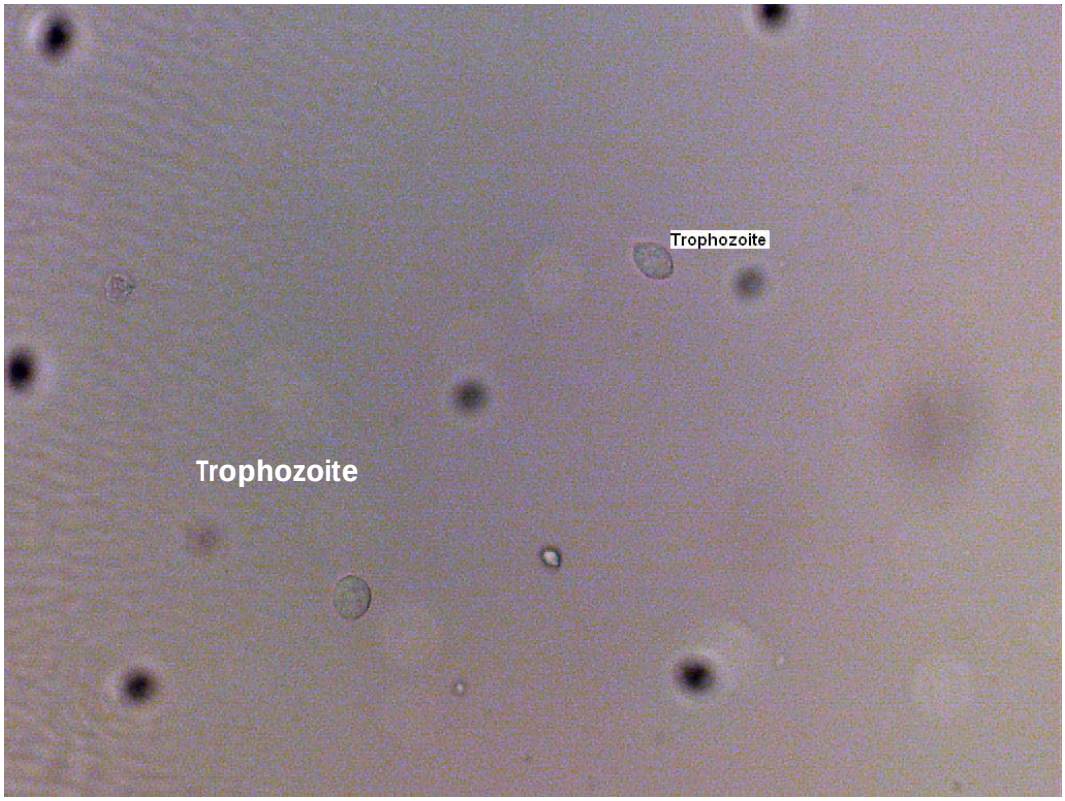
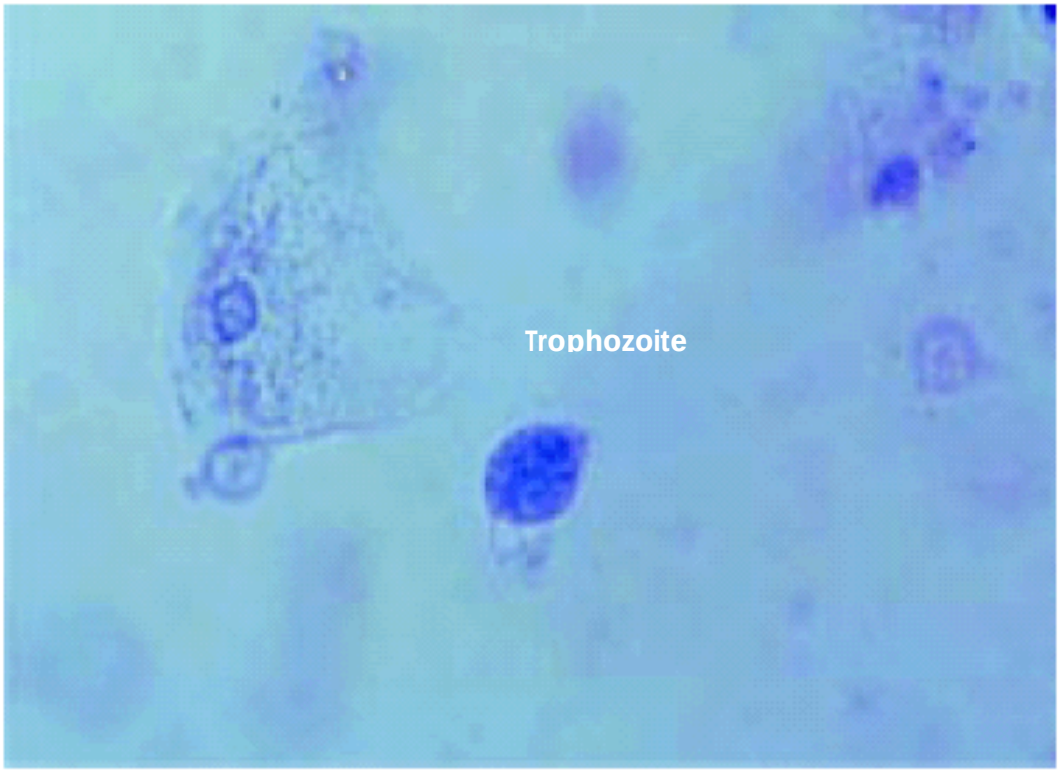


Fig 9: Methylene blue stained preparation trophozoite of *Trichomonas vaginalis* in a culture positive sample



positivity within 48 hours. Among these only one was positive by wet mount preparation as well and that sample showed positivity in culture by 24 hours. A photograph of the un-inoculated and inoculated modified Diamond's media is shown in **figure 10**

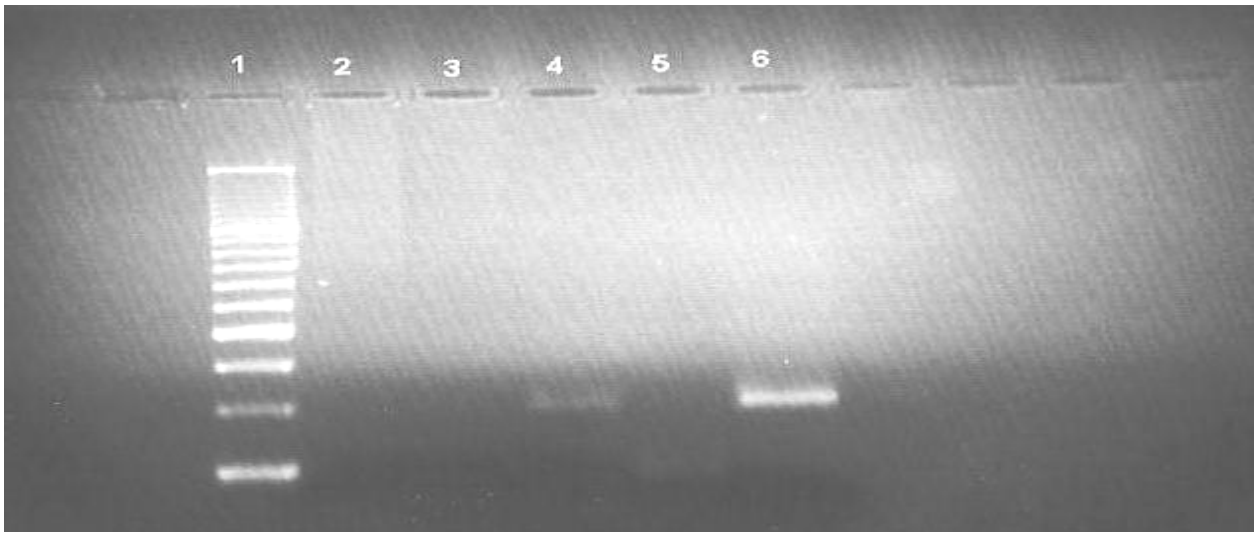
Ten (5.03%) samples were found to be positive by the non- nested PCR assay. All the 6 culture positive samples were also positive by the PCR. One of the culture positive samples, which were also positive by wet mount preparation, was negative by the PCR when first tested. However, PCR was repeated with 1/10 and 1/100 diluted DNA input, the 1/100 diluted sample showed a positive result. This negative PCR result in the undiluted sample and the 1/10 diluted sample may be due to the template excess. A gel picture showing the PCR in different dilutions for the sample which was wet mount and culture positive is shown in **Figure 11**.

The performance of the culture and the non nested PCR assay was compared using the Kappa coefficient analysis. There was a significant observed agreement of 98% (kappa coefficient of 0.74) between the PCR and culture (Z value 10.79, p value <0.001). The agreement of the PCR assay findings and culture results in the prospectively collected samples are shown in **Table 6.**

There were 6 culture negative samples which were positive by PCR when tested for the first time. On adjudication of these 6 discrepant samples by repeating the PCR assay, 4 culture negative but PCR positive samples were repeatedly positive. Any samples which showed positivity twice or more were taken as positive for the analysis. The results of the PCR assays run in triplicate for the 6 discrepant samples in culture and PCR with the final results are shown in **Table 7.**

Fig10: A typical photograph of the un-inoculated and inoculated modified Diamond's media

Figure 11: A gel picture showing the PCR positivity in different dilutions for the sample showing wet mount and culture positive.



Lane 1	Molecular Weight marker
Lane 2	concentrated sample
Lane 3	1/10 dilution of the concentrate
Lane 4	1/100 dilution of the concentrate
Lane 5	Negative control
Lane 6	Positive control

Table 6: Agreement of PCR assay findings and culture results in the prospectively collected samples

		***T.Vaginalis* Culture status**	
		POSITIVE	**NEGATIVE**
PCR	**POSITIVE**	6*	4
	NEGATIVE	0	188

** One of the culture and wet mount positive sample amplified only after testing a 1/100 diluted DNA (template excess)*

Observed agreement	0.98
Chance agreement	0.92
Kappa	0.74
SD error of Kappa	0.0686
Z	10.79
P value	0.001

Table 7: Results of the adjudication of discrepant samples (n=6) by repeat PCR testing

Patient ID	Culture	PCR1	PCR2	PCR 3	Final Result
TV 73	Negative	positive	Negative	Positive	**Positive**
TV 76	Negative	Positive	Positive	Positive	**Positive**
TV 214	Negative	Positive	Negative	Positive	**Positive**
TV 230	Negative	positive	Negative	Negative	Negative
TV 263	Negative	positive	Negative	Negative	Negative
TV 306	Negative	positive	Positive	Positive	**Positive**

Proportion of samples positive (5.5%) by any one tests in symptomatic (n=55) individuals were higher (4.9%) than asymptomatic individuals (n=143). However, this difference was not significant ($p = < 0.05$). These data is shown in **Table 8.**

Considering culture as gold standard the sensitivity of the wet mount and the PCR were analyzed. The sensitivity of the wet mount was only 16.6% with 100% specificity. This accuracy indices of the wet mount compared to the broth culture is shown in **Table 9.**

The non nested PCR assay had a sensitivity of 100% and a specificity of 98% when compared to the broth culture. The accuracy indices calculated for the PCR based on the culture is shown in **Table 10.**

The frequency of *Trichomonas vaginalis* by PCR in the HIV infected woman in our population was 5.05%. The percentage positivity for the different assays used in the study is shown in **Table 11.** The profile of the 10 *Trichomonas vaginalis* positive women by any one test with other available relevant tests is shown in **Table 12.**

Table 8: Laboratory findings in HIV infected women who presented with vaginal discharge or no abnormality

	No. of patients	Wet mount Positive	Culture Positive	PCR Positive	Any one test Positive
Symptomatic	55 (27.7%)	0(0%)	2 (3.6%)	3 (5.5%)	3 (5.5%)
Asymptomatic	143 (72.22%)	1 (0.6%)	4 (2.7%)	7 (4.9%)	7(4.9%)
Total	198	1	6	10	10

Table 9: The accuracy indices calculated for the wet mount preparation based on the culture results

		T.vaginalis Culture status	
		POSITIVE	**NEGATIVE**
T.vaginalis **WET MOUNT**	**POSITIVE**	1	0
	NEGATIVE	5	192

SENSITIVITY	16.7% (0.9 – 63.5) [#]
SPECIFICITY	100% (97.6 – 100)
POSTIVE PREDICTIVE VALUE	100% (5.5 – 100)
NEGATIVE PREDICTIVE VALUE	97.5% (93.9 – 99.1)

[#] Figures in parentheses are the 95 % confidence interval values

Table 10: The accuracy indices calculated for the PCR based on the culture findings

		***T.vaginalis* Culture status**	
		POSITIVE	**NEGATIVE**
T.vaginalis **PCR ASSAY**	**POSITIVE**	6*	4
	NEGATIVE	0	188

** One of the culture and wet mount positive sample amplified only after testing a 1/100 diluted DNA (template excess)*

SENSITIVITY	100%	(51.7 – 100) [#]
SPECIFICITY	97.9%	(94.4 – 99.3)
POSTIVE PREDICTIVE VALUE	60%	(27.4 – 86.3)
NEGATIVE PREDICTIVE VALUE	100%	(97.5 – 100)

[#] Figures in parentheses are the 95 % confidence interval values

Table 11: Laboratory findings in the samples obtained from HIV infected women

Type of tests	No of positives	Percentage
Wet mount	1	0.5
Culture	6	3.0
PCR	10	5.05
Both wet mount and culture positive	1	0.5
Both culture and PCR positive	6	3.0
All three test positive	1	0.5
Any one test positive	10	5.05

Table 12: The profile of the ten *Trichomonas vaginalis* positive (by any one test) individuals with other available relevant test results

Sl. No	Patient ID	Age (years)	Area	Symptom	WP	Culture	PCR	HbsAg	VDRL	HCV
1	TV24	30	TN	asymptomatic	+	+	+	-	-	-
2	TV 73	35	TN	asymptomatic	-	-	+	NA	NA	Neg
3	TV76	33	AP	asymptomatic	-	-	+	Neg	NR	NA
4	TV200	33	TN	***Symptomatic***	-	+	+	Neg	NR	Neg
5	TV204	25	TN	asymptomatic	-	+	+	Neg	NR	Neg
6	TV214	27	TN	asymptomatic	-	-	+	Neg	NR	Neg
7	TV232	48	TN	asymptomatic	-	+	+	Neg	NR	Neg
8	TV258	25	AP	asymptomatic	-	+	+	Neg	NR	Neg
9	TV286	28	TN	***Symptomatic***	-	+	+	Neg	NR	Neg
10	TV306	30	TN	***Symptomatic***	-	-	+	Neg	NR	Neg

#NA – not available

NR- Non Reactive

Neg- Negative

DISCUSSION

6. DISCUSSION

Trichomoniasis caused by *Trichomonas vaginalis* is the common non viral sexually transmitted disease among humans (111). The annual incidence of this disease globally as estimated by WHO is 170 million (114). In this study reported here a PCR targeting the adhesion and Beta tubulin genes of *T. vaginalis* was standardized for the detection of the parasite in vaginal swab samples. The usability of this PCR was compared with culture and wet preparation of prospectively collected vaginal swab samples from HIV infected women.

There are number of tests that can be used for the diagnosis of Trichomoniasis. These include vaginal wet preparation, vaginal swab culture and vaginal swab PCR. Apart from vaginal swabs, urine can be also used as a specimen for the diagnosis by any one of the mentioned tests (54, 91). In a randomized community based study done in Kenya, among 675 women using self collected vaginal swabs and centrifuged urine samples, *T.vaginalis* was detected in 17.9% of the women using vaginal swab samples. However, the positivity was only 3.4% by using centrifuged urine as sample. The culture of centrifuged urine showed a sensitivity of 17% with a specificity of 99.6% (71). In another study done at the University of Alabama in Birmingham, among 190 women attending a STD clinic, compared the usability of vaginal swabs and urine specimen for the detection of *T.vaginalis* using visualized from any wet preparation or culture as gold standard. Compared to the gold standard, vaginal swab culture showed a sensitivity of 94.3% while the sensitivity of urine sample was 60.4% (54).

Thus in both the above studies, urine specimen used for diagnosis of Trichomoniasis both in community as well as hospital setting showed a poor sensitivity. Therefore in our study vaginal swab was used for the detection of *T.vaginalis*.by the three methods carried out.

The most commonly used test for the diagnosis of *T. vaginalis* is the wet preparation. The sensitivity of wet preparation as compared to culture varies from 30% – 58% (62, 91). The sensitivity of wet preparation shown to be less in asymptomatic individuals compared to symptomatic individuals (92). The wet preparation has a number of limitations like the sensitivity is highly dependent on the expertise of the individual taking the reading, prompt transport of the specimen and processing of the sample required by the laboratory before the organism loses its motility or lyses (62). One another issue is that the size of the trichomonad is approximately the same as that of a lymphocyte or a small neutrophil and could be confused with this. More over when the organism is not motile, trichomonads can be difficult to differentiate from the nucleus of a vaginal epithelial cell. Motility of *T. vaginalis* is very much dependent on the temperature at which the specimen is stored. At room temperature the organism will remain alive for more than 6 hours but as the time passes the organism's motility becomes significantly attenuated.(31) However, there are several advantages as well like the test is rapid and most cost effective. Though the sensitivity of the wet preparation can be increased by making the preparation at the site of collection of sample it is still sub-optimal when compared to other techniques (31). In our study the sensitivity of wet mount was only 16.7% when compared to culture even after carrying out the examination of the wet preparation at the site of the sample collection by the technicians in both the ID clinics chosen for the study. Based on the wet mount preparation the frequency of *T.vaginalis* in our study population was only 0.5%. The positive predictive value was 100% with 95% CI of 5.5 to 100.

In a study conducted in Birmingham, Alabama University, among 190 women attending the STD clinic, the prevalence of trichomoniasis was 28%. Wet preparation was done on vaginal swabs and the sensitivity of wet mount was 58.5%.as compared to culture. Sood et al (2007) has

compared the wet preparation and In pouch culture with samples from women presenting with genitourinary symptoms attending a primary health center clinic in Haryana, India. Compared to the culture the sensitivity of the wet preparation was 55%. As reported previously, compared to culture the sensitivity of the wet preparation is quite poor in our study as well. One of the disadvantages with this low sensitivity of detection of *Trichomonas vaginalis* among HIV infected women by the routine wet preparation is that many infected women may not receive appropriate treatment otherwise with metronidazole. This will lead to complications in infected individuals, transmission to sexual partners, and also can even increase the transmission of HIV. These findings suggest the need for a more accurate diagnostic method for trichomoniasis.

Broth culture technique using Diamond's medium is considered as the 'gold standard' for the diagnosis of Trichomoniasis. The minimum inoculum size required for a positive result is about 102 organisms/ ml and the growth of the organism is easy to interpret (100). While the number of organism required for a wet preparation is about 10^4 organism/ml (31, 93). The standard culture broth used for culture of *T. vaginalis* is the Diamond's TYI medium in glass tubes (25,31). The sensitivity varies from 85-95% (28, 62, 98, 100). However, this too has its own limitations. It requires special media, results are available within 2-7 days and a microbiologist is required for the test (86). Another disadvantage is the contamination with bacteria of vaginal flora even with broth cultures spiked with antibiotics. Normally Diamond's media uses horse serum for the growth of the organism. However, the media we used for culture contained sheep sera instead of horse sera. This modification hasn't changed the sensitivity of the medium in our laboratory (29, 67). We also used Diamond's medium with penicillin and streptomycin but without amphotericin B. There are several studies which demonstrated that *T. vaginalis* can be cultivated successfully without amphotericin B (117). However, in our study few of the samples

showed growth of Candida within in 24 hours of incubation and by 48 hours there was over growth of candida. This may have prevented the appreciation of the typical motility of *T.vaginalis.* Our experience form this study suggest that it would be better to add amphotericin B or any other antifungal agents in the Diamond's media for the culture of *Trichomonas vaginalis.*

There is also a modified and simplified culture system commercially available, the In Pouch system (Biomed Diagnostics, USA). In this culture system the specimen is put into a two-chambered bag, allowing sampling for immediate wet mount microscopy and incubation for culture (15). Sood et al (2007) has found this In Pouch *Trichomonas vaginalis* culture system is a simple, cost-effective and a sensitive method for diagnosing *T. vaginalis* (93). Although the combination of culture and wet mount examination remains the standard approach for detecting *T. vaginalis* in patient samples there is no gold standard for confirmation (49). There are several studies which looked at the usability of polymerase chain reaction (PCR) for the detection *T. vaginalis*. PCR was shown to be more sensitive (80-100%) (54, 86, 90, 112) than culture and wet preparation.

A number of primers have been described for PCR vaginal swab as the specimen (54, 61, 62, 80). In a study done in STD clinic at Seattle among symptomatic and asymptomatic women, primer designed for the adhesisn gene of *trichomonas vaginalis* (TVA5/TVA6) was able to detect all the 24 isolates as compared to all the other three sets of primers namely target sequence T17rp (TR7/8), target sequence T9p (TR5/6) and ferreoxdine gene (FE1/2). In our study also we used TVA5/TVA6 primer set. This primer set amplifies a 102bp genomic sequence termed as A6p. This area is a much conserved segment of the adhesisn gene and hence the PCR using primer set of TVA5/TVA6 was proved to be highly selective for a wide range of *T.vaginalis* isolates (86).

In an another study conducted among the female sex workers attending the STD clinic Institute of Tropical Medicine, Belgium, where three sets of primers were evaluated using vaginal swab as sample. The primer sets used were, TVA5/TVA6, one amplifying a portion of beta tubulin gene (BTUB9/2) and 312bp sequence to amplify repetitive DNA specific to *T.vaginalis* (TVK3/7). The sensitivity and specificity of the PCR with primer set BTUB9/2 was 77.1 and 96%, with TVA5/TVA6 it was 64% and 99.7% respectively. The sensitivity and the specificity with the TVK3/7 primer set was 88% and 97% respectively. According to this study, TVA5/6 was highly specific, were as TVK3/7 showed a high sensitivity (104). A study done among the women attending the epidemiology clinic in Maryland again on vaginal swabs showed a sensitivity of 97% and specificity of 98% with the primer set of BTUB9/2 (62).

Another study done in Netherlands, again with vaginal swabs as sample, and the primer set TVK3/7 detected 87% of the culture positive. These PCR assay also detected 12 cases by PCR alone. TVA5/6 being highly specific for *trichomonas vaginalis* was used for the confirmation of these positives, and 9 cases were confirmed positive. All the above mentioned primers showed different sensitivity and specificity in different studies. This variation may be due to the variation of the strain circulating in any one region (90).

In the study reported here two sets of primers TVA5/6 and BTUB9/2 were selected based on their sensitivity and specificity. The primer TAV5/6 which is highly specific to detect the adhesion gene was chosen along with another primer BTUB9/2 to detect beta tubulin gene also shown to have very high sensitivity and specificity. While standardization the results with primer set of TAV5/TAV6 were found to be better in detecting different dilution of the broth culture when compared to the BTUB2 primers. Therefore it was decided to use the primer set of TVA5/TVA6 for the PCR amplification.

However, the PCR with TVA5/TVA6 using on the positive and negative cultures, with commercial regents using recombinant Taq polymerase (Fermentas Life Sciences, USA), it showed a sensitivity of (43.3%) compared to culture positive samples. The specificity of this primer set was found to be good (98%). The specificity depends on the annealing temperature, with higher temperatures favoring higher specificity. In this study reported we also used the touchdown protocol of PCR to increase the specificity of the assay. This favored the amplification of targeted copies of DNA amplified during early cycles at higher annealing temperatures and eliminating spurious products. Since the earlier studies showed a sensitivity of > 90% for this primer set comparatively far better than ours we decided to change the PCR reagents used.

In order to improve the sensitivity of the PCR assay we further used HotStarTaq Master Mix reagent (Qiagen, Hilden, Germany) for doing the assay, using the same set of primers. This Master Mix contains HotStarTaq DNA Polymerase. HotStarTaq DNA polymerase has been used to do touchdown enzyme time PCR for *C. trachomatis, C. psittaci* and *C. pneumoniae* using 16-Sand 16-s 13-s Spacer r-RNA gene. The sensitivities shown by this PCR was very similar to that shown by AmpliTaq Gold suggesting that HotStarTaq ampliTaq Gold can be used as an alternative (61). Like the AmpliTaq Gold, the HotStarTaq also not active until heated to 95°C. The use of this enzyme was also able to avoid erroneous amplification of DNA products due to nonspecific annealing of primers at lower temperatures.

In our study, the use of HotStarTaq Master Mix in place of conventional PCR reagents (recombinant DNA Taq Polymerase) showed a marked improvement in the sensitivity of the assay. The sensitivity improved from 43.3% to 86.6% compared with culture i.e. there was an improvement in sensitivity by 100% which is quite significant. Specificity of the TVA5/TVA6

remained the same with this reagent. These four culture positive samples were tested repeatedly and found negative. In order to rule out any template excess that may inhibit the PCR, extracted DNA from these samples were retested in dilution of /10 and 1/100. However, the result was same. In this study the PCR with TVA5/TVA6 primer set failed to detect 4 culture positive samples. Reason could be attributed to the sensitivity of the primer used or those strains may be a variant that prevented the primers annealing to the DNA. It is possible that the use of another primer set like BTUB9/2 to detect another region of the *T.vaginalis* genome could have detected these culture positives. There are several studies for other organisms which have demonstrated that detection of more than one region by PCR can increase the sensitivity of the assay (62).

In the study reported here we collected the samples *i.e* vaginal swabs in 1 ml sterile normal saline squeezed out the swab and stored it till extraction at -20˚C. Pillay *et al* (2007) has also shown the successful amplification of DNA from vaginal washes with normal saline (80). A total of 10 samples were found positive by the PCR assay used. In our study all the 6 culture positive samples were also positive by the PCR assay. However, one of the culture positive samples became positive only after repeating the PCR with a 1/100 diluted DNA input. This particular sample was also positive by the direct wet mount preparation. This indicated a larger number of parasites in the original sample. Hence, the lack of amplification in the undiluted and 1/10 diluted sample may be due to template DNA excess. The template excess may cause chelation of $Mgcl_2$ added to suboptimal level for the results for the PCR reactions.

Six culture negative samples were found to be positive by PCR during the initial run. In order to confirm the true results all the 6 samples which showed discrepant results were repeat tested in duplicate by the PCR assay. A concordant result of two or more times in the triplicate run was considered as the final result. Four of the 6 culture negative samples were repeatedly positive in

the PCR assay and those samples were considered as true positive as the primer set TVA5/TVA6 was highly specific. However, the ideal way to confirm the true positivity is to sequence the product and see it matches with the expected nucleotide sequence or carry out another PCR for the amplification of a different region. Compared to the culture the PCR assay used in our study was found to have significant observed agreement ($Z = 10.49$, $p < 0.001$) with a Kappa coefficient 0.74. Moreover the assay was able to detect 4 additional samples than culture.

As for the quality checkup of the prepared media, as recommended by the National committee for clinical laboratory Standards guidelines, 5% of media in each batch must be inoculated with reference strain ATCC30001 of *T.vaginalis.* For this study we could not procure the reference strain of *T.vaginalis* due to the inability to maintain the strains in our laboratory. Hence, for the media quality checking we inoculated three tubes of media per batch (each batch 15 tubes) with the positive culture we obtained with the previous batch of media and checked for growth by preparing wet mounts for 7 days. However, we had three batches of media for which we could not check the quality as the previous batches did not have any positive samples and laboratory did not have any facility for storage in a viable form of the previous positive strains. There is a possibility that we would have missed some positive samples due to this reason. We had picked up 4 samples as positive by PCR, which were negative by culture. The quality of the media in the batches which picked up these positives had been checked and was found to be satisfactory. This makes us believe that the quality of those three batches of media was also satisfactory.

The prevalence of *T.vaginalis* varies in different population. This depends on their social and demographic profile. A study conducted by Mathai *et.al* (1998) among 300 women coming for antenatal check up in a tertiary care set up in South India showed that 6% of these women had *T.vaginalis* infection. Among the positive women only 3(7%) were symptomatic (67). In another

study among 232 women attending antenatal clinic at Denver, 14.66% were detected to have *T.vaginalis* infection (22). In a multicentre study by Uneke et al (2005) among 502 women attending antenatal clinic in South–Eastern Nigeria in the year 2005 , the prevalence of *T.vaginalis* was shown as 6.9%.The prevalence was seen highest (3.7%) among 20-25yrs and lowest (1.9%) among 26-30yrs of age (106).

In a community based cross-sectional study from a rural area done by Prasad et al (2005) among 451 married women between 16-22 yrs of age, in Tamil Nadu, 13% of women had *T. vaginalis* infection. Among the positives 70 % were asymptomatic and 30% were symptomatic (83). A multicentric community based study of sexually transmitted disease in Surat, India among 102 women showed 34.3% prevalence of *T.vaginalis.* It was also found that among 16 women who had symptoms 75% women had *T. vaginalis* infection while among the remaining asymptomatic women 26.6% had trichomoniasis (17). Study done by Bhatt *et al* (1997) in the gynecology out-patient clinic of a tertiary care set up in Mumbai, India among 500 women with symptoms, infertility or pregnancy as well as asymptomatic women showed that 85 (17%) of these women had Trichomoniasis. Among whom 61 women were symptomatic, 18 were pregnant and 6 women were infertile (13). Retrospective analysis trends of STD in clinics at Chengalpattu, Tamil Nadu over a period of 7yrs (1988-1994) reported a total attendance of 4549 patients with 3621 (79.6%) males and 928 (20.4%) females. Among the females 17.2% were detected to have *Trichomonas vaginalis* infection.(49) Desai *et al*. (2003) had investigated 124 sex workers in whom the prevalence of syphilis, gonorrhea, chlamydial infection and trichomoniasis were looked at. All these women belonged to the ages of 16-50 yrs, and 58.5% of these were

asymptomatic. Among the symptomatic 12.7% women had vaginal discharge and 14.4% women were confirmed to have trichomoniasis (20).

In a case controlled study reported from Congo, among the HIV infected and uninfected mothers 18.6% of HIV infected women and 10.2% of HIV uninfected women were shown to have *T. vaginalis* infection (101). In another study done in USA among the HIV infected individuals (228 seropositive men and 340 seropositive women) 9% had trichomoniasis, 6% gonorrhea, 4% chlamydia and 22% other STI's. The mean age among the sexually active women with STI's was reported as 38.3 and about 17% of these women had symptoms of vaginal discharge (42). In another study in a STD clinic at New York among 1285 HIV infected individuals and HIV non infected women but with high risk behavior 12% and 10% of frequency reported for trichomoniasis respectively (19). Compared to these reports the frequency of *T.vaginalis* in HIV seropositive Nigerian women were found to be very high (99). *T.vaginalis* infection was highly prevalent (32.6%) in women in age group of 26yrs – 30yrs, and least (18.8%) prevalent among women in age group of 20yrs -25yrs and more than 40yrs (106). Another report from Nigeria demonstrated the prevalence of *T. vaginalis* in different age groups and were as follows, 30% in 5-15yrs, 46.6% between 16-21 yrs, 41.2% between 28-33 yrs, 28.9% between 34-39 yrs, 27.7%between 22-27yrs about 10% in 40-51yrs and 16-21yrs. *T.vaginalis* infection was not seen in 52-57 yrs age group individuals (76).

In our study 204 women were recruited. These women were chosen on their first visit to either of the two infectious disease clinic selected for the study. A good number of patients were either referred from other hospitals or from other clinics in this hospital, to the infectious disease clinic. Two such patients, who were detected to be HIV seropositive in a center elsewhere, were

confirmed negative on repeating the HIV test in our hospital. These two women were excluded from the study. Another four patients who though HIV positive could not be enrolled in the study because, for two of them saline swabs for PCR were not taken by the clinician, while there was a spillage of the culture media with the samples taken in 2 cases during transport. Hence, only 198 HIV seropositive women were included in the study and all three tests were done on vaginal swabs taken from them. Geographically, there was an unequal representation of patients from both Northern and Southern part of India with predominance from South India as the hospital is situated in the south.

Out of 198 HIV positive females included in our study, there were only 6 patients (3%) who were positive for *T. vaginalis* infection by culture. Most of these patients were in the age group of 25-33 years. This is quite comparable with the age distribution in the above studies .The age group between 15-45 yrs is considered as the reproductive age and also during this period females are sexually active. All the women included in our study were married. Majority (73%) of our study participants were asymptomatic women. The proportion of women positive for *Trichomonas vaginalis* by any one test in our population was higher among the symptomatic individuals (5.5%) compared to asymptomatic women (4.9%) but the difference was not statistically insignificant ($p > 0.05$). Our results were in similar to studies done in Tamil Nadu, Mumbai and Surat where higher percentage of *T. vaginalis* infection was seen among the symptomatic women (17, 20, 67). Four (67%) out of 6 women tested positive by culture and seven (70%) out of 10 women tested positive by PCR assay compared were asymptomatic. This result is very similar to the study by Mathai *et al* (1998) from the same institution where only 3 (17%) of 18 women found positive for *T. vaginalis* in antenatal clinic were symptomatic. It is possible that women who came to our hospital who had symptoms suggesting *T.vaginalis*

infection would have been treated elsewhere and were negative when tested here. Moreover as the patients who had tested positive for *T. vaginalis* were mostly asymptomatic would not have felt the need to seek treatment, and were accidently detected positive on routine checkup. As discussed earlier the presence of one STI can cause transmission of other STI's, moreover an asymptomatic individual may not feel the need for investigation and treatment, an efficient and sensitive method of screening such patients is needed.

Our study has shown the frequency of trichomoniasis in HIV infected women as 5.05 % with PCR or any one assay positive. We expected a higher frequency of *Trichomonas vaginalis* among the HIV infected women. However, this frequency is almost similar (6%) to the frequency seen among women in antenatal clinic but lower (13%) than reported from a rural community and STI clinic attendees of neighboring district of Tamil Nadu (49, 67, 83). The possible reason for this low prevalence could be attributed to, the selection of patients for the study. Since the study setups were in tertiary care centre and relatively expensive, only patients who can afford the management may come to the hospital. Another reason may be the use of metronidazole prescribed by the general physicians and later coming to a tertiary care center like our hospital. The study done in high risk groups with other sexually transmitted infections would have possibly shown a different picture.

In conclusion our study clearly showed that the PCR assay using the TAV5/TAV6 primer set is a highly sensitive method for the detection of *Trichomonas vaginalis* compared to the culture. The frequency of the infection in HIV infected woman was 5.05% by any one positive test. It is important to screen the high risk group individuals like HIV positive women for *Trichomonas vaginalis* routinely with highly sensitive techniques like PCR as most of the individuals are asymptomatic and may not receive appropriate treatment otherwise with metronidazole. Lack of

timely management of the infection may lead to complications in infected individuals and also can increase the transmission of HIV to partners. Testing algorithm should be wet mount and if negative test by PCR as it is rapid compared to culture which takes 7 days.

SUMMARY AND CONCLUSIONS

7. SUMMARY AND CONCLUSION

Trichomoniasis caused by the protozoan parasite *Trichomonas vaginalis* is the most common non-viral sexually transmitted infection. In this study reported here a PCR targeting the adhesion and Beta tubulin genes of *T. vaginalis* was standardized for the detection of the parasite in vaginal swab samples. The usability of this PCR was compared with culture and wet preparation of prospectively collected vaginal swab samples from HIV infected women attending infectious disease clinics.

- In the standardization of PCR the primer set TAV5/TAV6 targeting the adhesin gene of *Trichomonas vaginalis* was found to have better performance in experiments on lower limit of detection than the BTUB9/2 primer set targeting the Beta tubulin
- The HotStar Taq Master Mix (Quaigen, Hilden, Germany) for the PCR assay has significantly improved the sensitivity of the PCR from 43.3% to 86.7% i.e., an 100% increase compared to the regular Taq polymerase (Fermentas, Life Sciences, Canada, USA). The specificity (97.6%) of the PCR assay was same with both the reagents used.
- Majority of the participants (91%) in the prospective study were from South India. Among the 198 study participants 140 were from state of Tamil Nadu.
- There were 55 symptomatic and 143 symptomatic participants.
- Mean age of the study participants was 33.6 yrs (21-52 yrs) with 84% of the individuals in the sexually active age group of 20-39.
- Majority of the study patients were housewives 180(91%)

- Among 198 HIV seropositive women only 1 (0.05%) tested positive for *Trichomonas vaginalis* by wet mount preparation. The sensitivity of wet mount as compared to culture was only 16.7%.
- Six (3%) of the 198 samples showed positivity in modified Diamond's culture medium.
- Ten (5.05%) samples were found to be positive by the non nested PCR assay. All the culture positive samples were detected by the PCR assay. However, one sample which was positive by culture and wet mount preparation showed positivity in PCR with 1/100 diluted DNA input. The negative PCR result in the undiluted and the 1/10 diluted sample may be due to the template excess.
- Compared to broth culture, the non nested PCR assay showed 100% sensitivity in the prospective study. Moreover the assay was able to detect 4 additional samples than culture.
- There was a significant observed agreement of 98% (kappa coefficient of 0.74) between the PCR and culture (Z value 10.79, p value <0.001) in the prospective study.
- The proportion of women positive for *Trichomonas vaginalis* by any one test in our population was higher among the symptomatic individuals (5.5%) compared to asymptomatic women (4.9%). This difference was not statistically significant >0.005. Four (66.6%) out of 6 women tested positive by culture and 7 (70%) out of 10 women tested positive by PCR assay were asymptomatic.
- The frequency of trichomoniasis in the HIV infected women of our study population was 5.05 % by any one test.

- The PCR assay used in this study showed good sensitivity compared to culture in Diamond's media in the prospective study. Moreover the assay also detected 4 additional culture negative samples. The assay is very rapid compared to culture.
- The testing algorithm should be wet mount which if negative, PCR assay should be done since it is rapid and takes a less duration.

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APPENDIX

PROFORMA

Study No:

Name of the Patient:

Age:

Hospital Number:

Residence:

Occupation:

Symptoms:

Current Clinical Diagnosis:

Treatment History:

Test to rule out other infection: HIV HCV HbSAg VDRL

Date of Sample collection: **Signature of physician/Researcher**

Serial no	Code no	Sample no	Hospital no	Name of Patients	wet mount	Culture	PCR 1
1	HP101	TV258	132526	Parameshwari	**POSITIVE**	**POSITIVE**	NEGATIVE
2	HP102	TV270	431497	Jeyakodi	NEGATIVE	NEGATIVE	**POSITIVE**
3	HP103	TV267	110668	Kalyani	NEGATIVE	NEGATIVE	NEGATIVE
4	HP104	TV395	103192	Jothylakshmmi	NEGATIVE	NEGATIVE	NEGATIVE
5	HP105	TV245	191152	Sumathy	NEGATIVE	NEGATIVE	NEGATIVE
6	HP106	TV181	212504	Nadhiya Manoharan	**POSITIVE**	**POSITIVE**	NEGATIVE
7	HP107	TV304	325662	Adilakshmi	**POSITIVE**	**POSITIVE**	NEGATIVE
8	HP108	TV650	357315	Thilagavathy	**POSITIVE**	**POSITIVE**	NEGATIVE
9	HP109	TV333	473677	Shylaja	**POSITIVE**	**POSITIVE**	**POSITIVE**
10	HP110	TV6	404986	Jothy	NEGATIVE	NEGATIVE	NEGATIVE
11	HP111	TV341	461274	Thanjammal	NEGATIVE	NEGATIVE	NEGATIVE
12	HP112	TV335	440078	Sudha	**POSITIVE**	**POSITIVE**	**POSITIVE**
13	HP113	TV306	333524	Amudha	NEGATIVE	NEGATIVE	NEGATIVE
14	HP114	TV303	325776	Anabarasi	**POSITIVE**	**POSITIVE**	**POSITIVE**
15	HP115	TV302	325444	Lakshmi	NEGATIVE	NEGATIVE	NEGATIVE
16	HP116	TV550	263071	Meena	**POSITIVE**	**POSITIVE**	**POSITIVE**
17	HP117	TV537	16169	Jayanthi	NEGATIVE	NEGATIVE	NEGATIVE
18	HP118	TV450	640704	Girija	NEGATIVE	**POSITIVE**	NEGATIVE
19	HP119	TV293	132156	Manjula	**POSITIVE**	**POSITIVE**	NEGATIVE
20	HP120	TV301	325537	Deepa	NEGATIVE	NEGATIVE	NEGATIVE
21	HP121	TV281	104570	Elyarani	**POSITIVE**	**POSITIVE**	NEGATIVE
22	HP122	TV278	104950	Thamaramani	NEGATIVE	NEGATIVE	NEGATIVE
23	HP123	TV613	492940	Shyamalamani	NEGATIVE	NEGATIVE	NEGATIVE
24	HP124	TV320	324955	Kalaiselvi	NEGATIVE	NEGATIVE	NEGATIVE
25	HP125	TV183	161282	Pushpa Ravizuman	NEGATIVE	**POSITIVE**	**POSITIVE**
26	HP126	TV318	325054	Lakshmi	NEGATIVE	NEGATIVE	**POSITIVE**
27	HP128	TV232	118830	Chellatalini	NEGATIVE	**POSITIVE**	**POSITIVE**
28	HP129	TV311	323478	Selvi	NEGATIVE	NEGATIVE	NEGATIVE
29	HP130	TV276	104968	Remya	**POSITIVE**	**POSITIVE**	**POSITIVE**
30	HP131	TV336	432683	Poonkodi	NEGATIVE	NEGATIVE	NEGATIVE
31	HP133	TV418	534468	Jayasudha	NEGATIVE	NEGATIVE	NEGATIVE
32	HP134	TV100	200907	Suthasumitra	**POSITIVE**	**POSITIVE**	**POSITIVE**
33	HP135	TV339	450561	Sumitha	NEGATIVE	NEGATIVE	NEGATIVE
34	HP136	TV163	310936	Thenmozahi	**POSITIVE**	**POSITIVE**	NEGATIVE
35	HP137	TV289	110929	Sumathy	NEGATIVE	NEGATIVE	NEGATIVE
36	HP138	TV632	300902	Saridha	NEGATIVE	NEGATIVE	NEGATIVE
37	HP139	TV291	104404	Pushplatha	NEGATIVE	NEGATIVE	NEGATIVE
38	HP140	TV426	534594	Tamilarsi	**POSITIVE**	**POSITIVE**	NEGATIVE
39	HP141	TV275	104968	Sasikala	**POSITIVE**	**POSITIVE**	NEGATIVE
40	HP142	TV346	432704	Vijaylakshmi	NEGATIVE	NEGATIVE	NEGATIVE
41	HP143	TV315	325114	Manjula	NEGATIVE	NEGATIVE	NEGATIVE
42	HP144	TV529	373797	Lakshmi	NEGATIVE	NEGATIVE	NEGATIVE
43	HP145	TV354	474152	Poongavanam	**POSITIVE**	**POSITIVE**	**POSITIVE**

44	HP147	TV312	325108	Kokila	NEGATIVE	NEGATIVE	NEGATIVE
45	HP148	TV499	352322	Poornima	**POSITIVE**	**POSITIVE**	**POSITIVE**
46	HP149	TV612	481471	Lakshmi	NEGATIVE	NEGATIVE	NEGATIVE
47	HP150	TV531	461598	Manimeglai	NEGATIVE	NEGATIVE	NEGATIVE
48	HP151	TV51	324859	Sugna	**POSITIVE**	**POSITIVE**	**POSITIVE**
49	HP152	TV337	473547	Sudha	NEGATIVE	NEGATIVE	NEGATIVE
50	HP153	TV345	473680	Sythraiselvi	NEGATIVE	NEGATIVE	NEGATIVE
51	HP155	TV530	102505	Poonkodi	NEGATIVE	NEGATIVE	NEGATIVE
52	HP156	TV99	200907	Kamatachi	**POSITIVE**	**POSITIVE**	**POSITIVE**
53	HP157	TV321	333639	Priya	NEGATIVE	NEGATIVE	NEGATIVE
54	HP158	TV609	482167	Chandra	NEGATIVE	NEGATIVE	NEGATIVE
55	HP159	TV414	531390	Raleshwari	NEGATIVE	NEGATIVE	NEGATIVE
56	HP161	TV390	811545	Nirmala	NEGATIVE	NEGATIVE	NEGATIVE
57	HP163	TV1X	392523	Jamuna	**POSITIVE**	**POSITIVE**	NEGATIVE
58	HP164	TV325	324297	Mahasundari	NEGATIVE	NEGATIVE	NEGATIVE
59	HP165	TV460	599575	Kalaivani	**POSITIVE**	**POSITIVE**	**POSITIVE**
60	HP166	TV243	640429	Jeyamalini	**POSITIVE**	**POSITIVE**	**POSITIVE**
61	HP168	TV272	110972	Kalpana	NEGATIVE	NEGATIVE	NEGATIVE
62	HP169	TV346	432702	Vijaylakshmi	NEGATIVE	NEGATIVE	NEGATIVE
63	HP171	TV235	650459	Pushpa	NEGATIVE	NEGATIVE	NEGATIVE
64	HP172	TV646	313492	Deepa	NEGATIVE	**POSITIVE**	NEGATIVE
65	HP173	TV385	811398	Kalaiselvi	**POSITIVE**	**POSITIVE**	NEGATIVE
66	HP174	TV502	352314	Chitra	**POSITIVE**	**POSITIVE**	NEGATIVE
67	HP175	TV493	340200	Arputham	**POSITIVE**	**POSITIVE**	NEGATIVE
68	HP176	TV252	132531	Akila	NEGATIVE	NEGATIVE	NEGATIVE
69	HP177	TV305	333432	Devi	NEGATIVE	NEGATIVE	NEGATIVE
70	HP178	TV541	751338	Venda	NEGATIVE	**POSITIVE**	NEGATIVE

Serial no	Code no	Sample no	Hospital no	Name of Patients	Wet mount	culture	PCR2
1	HP101	TV258	132526	Parameshwari	**POSITIVE**	**POSITIVE**	**POSITIVE**
2	HP102	TV270	431497	Jeyakodi	NEGATIVE	NEGATIVE	NEGATIVE
3	HP103	TV267	110668	Kalyani	NEGATIVE	NEGATIVE	NEGATIVE
4	HP104	TV395	103192	Jothylakshmmi	NEGATIVE	NEGATIVE	NEGATIVE
5	HP105	TV245	191152	Sumathy	NEGATIVE	NEGATIVE	NEGATIVE
6	HP106	TV181	212504	Nadhiya Manoharan	**POSITIVE**	**POSITIVE**	**POSITIVE**
7	HP107	TV304	325662	Adilakshmi	**POSITIVE**	**POSITIVE**	**POSITIVE**
8	HP108	TV650	357315	Thilagavathy	**POSITIVE**	**POSITIVE**	**POSITIVE**
9	HP109	TV333	473677	Shylaja	**POSITIVE**	**POSITIVE**	**POSITIVE**
10	HP110	TV6	404986	Jothy	NEGATIVE	NEGATIVE	NEGATIVE
11	HP111	TV341	461274	Thanjammal	NEGATIVE	NEGATIVE	NEGATIVE
12	HP112	TV335	440078	Sudha	**POSITIVE**	**POSITIVE**	**POSITIVE**
13	HP113	TV306	333524	Amudha	NEGATIVE	NEGATIVE	NEGATIVE
14	HP114	TV303	325776	Anabarasi	**POSITIVE**	**POSITIVE**	**POSITIVE**
15	HP115	TV302	325444	Lakshmi	NEGATIVE	NEGATIVE	NEGATIVE
16	HP116	TV550	263071	Meena	**POSITIVE**	**POSITIVE**	**POSITIVE**
17	HP117	TV537	16169	Jayanthi	NEGATIVE	NEGATIVE	NEGATIVE
18	HP118	TV450	640704	Girija	NEGATIVE	**POSITIVE**	**POSITIVE**
19	HP119	TV293	132156	Manjula	**POSITIVE**	**POSITIVE**	NEGATIVE
20	HP120	TV301	325537	Deepa	NEGATIVE	NEGATIVE	NEGATIVE
21	HP121	TV281	104570	Elyarani	**POSITIVE**	**POSITIVE**	**POSITIVE**
22	HP122	TV278	104950	Thamaramani	NEGATIVE	NEGATIVE	NEGATIVE
23	HP123	TV613	492940	Shyamalamani	NEGATIVE	NEGATIVE	NEGATIVE
24	HP124	TV320	324955	Kalaiselvi	NEGATIVE	NEGATIVE	NEGATIVE
25	HP125	TV183	161282	Pushpa Ravizuman	NEGATIVE	**POSITIVE**	**POSITIVE**
26	HP126	TV318	325054	Lakshmi	NEGATIVE	NEGATIVE	NEGATIVE
27	HP128	TV232	118830	Chellatalini	NEGATIVE	**POSITIVE**	**POSITIVE**
28	HP129	TV311	323478	Selvi	NEGATIVE	NEGATIVE	NEGATIVE
29	HP130	TV276	104968	Remya	**POSITIVE**	**POSITIVE**	**POSITIVE**
30	HP131	TV336	432683	Poonkodi	NEGATIVE	**NEGATIVE**	**NEGATIVE**
31	HP133	TV418	534468	Jayasudha	NEGATIVE	NEGATIVE	NEGATIVE
32	HP134	TV100	200907	Suthasumitra	**POSITIVE**	**POSITIVE**	**POSITIVE**
33	HP135	TV339	450561	Sumitha	NEGATIVE	NEGATIVE	NEGATIVE
34	HP136	TV163	310936	Thenmozahi	**POSITIVE**	**POSITIVE**	**POSITIVE**
35	HP137	TV289	110929	Sumathy	NEGATIVE	NEGATIVE	NEGATIVE
36	HP138	TV632	300902	Saridha	NEGATIVE	NEGATIVE	NEGATIVE
37	HP139	TV291	104404	Pushplatha	NEGATIVE	NEGATIVE	NEGATIVE
38	HP140	TV426	534594	Tamilarsi	**POSITIVE**	**POSITIVE**	**POSITIVE**
39	HP141	TV275	104968	Sasikala	NEGATIVE	**POSITIVE**	**POSITIVE**
40	HP142	TV346	432704	Vijaylakshmi	NEGATIVE	NEGATIVE	NEGATIVE
41	HP143	TV315	325114	Manjula	NEGATIVE	NEGATIVE	NEGATIVE
42	HP144	TV529	373797	Lakshmi	NEGATIVE	NEGATIVE	NEGATIVE
43	HP145	TV354	474152	Poongavanam	**POSITIVE**	**POSITIVE**	**POSITIVE**

44	HP147	TV312	325108	Kokila	NEGATIVE	NEGATIVE	NEGATIVE
45	HP148	TV499	352322	Poornima	**POSITIVE**	**POSITIVE**	**POSITIVE**
46	HP149	TV612	481471	Lakshmi	NEGATIVE	NEGATIVE	NEGATIVE
47	HP150	TV531	461598	Manimeglai	NEGATIVE	NEGATIVE	NEGATIVE
48	HP151	TV51	324859	Sugna	**POSITIVE**	**POSITIVE**	**POSITIVE**
49	HP152	TV337	473547	Sudha	NEGATIVE	NEGATIVE	**POSITIVE**
50	HP153	TV345	473680	Sythraiselvi	NEGATIVE	NEGATIVE	NEGATIVE
51	HP155	TV530	102505	Poonkodi	NEGATIVE	NEGATIVE	NEGATIVE
52	HP156	TV99	200907	Kamatachi	**POSITIVE**	**POSITIVE**	**POSITIVE**
53	HP157	TV321	333639	Priya	NEGATIVE	NEGATIVE	NEGATIVE
54	HP158	TV609	482167	Chandra	NEGATIVE	NEGATIVE	NEGATIVE
55	HP159	TV414	531390	Raleshwari	NEGATIVE	NEGATIVE	NEGATIVE
56	HP161	TV390	811545	Nirmala	NEGATIVE	NEGATIVE	NEGATIVE
57	HP163	TV1X	392523	Jamuna	**POSITIVE**	**POSITIVE**	**POSITIVE**
58	HP164	TV325	324297	Mahasundari	NEGATIVE	NEGATIVE	NEGATIVE
59	HP165	TV460	599575	Kalaivani	**POSITIVE**	**POSITIVE**	**POSITIVE**
60	HP166	TV243	640429	Jeyamalini	**POSITIVE**	**POSITIVE**	**POSITIVE**
61	HP168	TV272	110972	Kalpana	NEGATIVE	NEGATIVE	NEGATIVE
62	HP169	TV346	432702	Vijaylakshmi	NEGATIVE	NEGATIVE	NEGATIVE
63	HP171	TV235	650459	Pushpa	NEGATIVE	NEGATIVE	NEGATIVE
64	HP172	TV646	313492	Deepa	NEGATIVE	**POSITIVE**	**POSITIVE**
65	HP173	TV385	811398	Kalaiselvi	**POSITIVE**	**POSITIVE**	**POSITIVE**
66	HP174	TV502	352314	Chitra	**POSITIVE**	**POSITIVE**	**POSITIVE**
67	HP175	TV493	340200	Arputham	**POSITIVE**	**POSITIVE**	**POSITIVE**
68	HP176	TV252	132531	Akila	NEGATIVE	NEGATIVE	NEGATIVE
69	HP177	TV305	333432	Devi	NEGATIVE	NEGATIVE	NEGATIVE
70	HP178	TV541	751338	Venda	NEGATIVE	**POSITIVE**	**POSITIVE**

MASTER CHART OF CULTURES SAMPLES BLINDED

(PCR ASSAY DONE WITH CONVENTIONAL METHOD)

Sl.No	Code no	Sample no	Hospital no	**wet mount**	**Culture**	**PCR 1**
1	HP101	TV258	132526	**POSITIVE**	**POSITIVE**	NEGATIVE
2	HP102	TV270	431497	NEGATIVE	NEGATIVE	**POSITIVE**
3	HP103	TV267	110668	NEGATIVE	NEGATIVE	NEGATIVE
4	HP104	TV395	103192	NEGATIVE	NEGATIVE	NEGATIVE
5	HP105	TV245	191152	NEGATIVE	NEGATIVE	NEGATIVE
6	HP106	TV181	212504	**POSITIVE**	**POSITIVE**	NEGATIVE
7	HP107	TV304	325662	**POSITIVE**	**POSITIVE**	NEGATIVE
8	HP108	TV650	357315	**POSITIVE**	**POSITIVE**	NEGATIVE
9	HP109	TV333	473677	**POSITIVE**	**POSITIVE**	**POSITIVE**
10	HP110	TV6	404986	NEGATIVE	NEGATIVE	NEGATIVE
11	HP111	TV341	461274	NEGATIVE	NEGATIVE	NEGATIVE
12	HP112	TV335	440078	**POSITIVE**	**POSITIVE**	**POSITIVE**
13	HP113	TV306	333524	NEGATIVE	NEGATIVE	NEGATIVE
14	HP114	TV303	325776	**POSITIVE**	**POSITIVE**	**POSITIVE**
15	HP115	TV302	325444	NEGATIVE	NEGATIVE	NEGATIVE
16	HP116	TV550	263071	**POSITIVE**	**POSITIVE**	**POSITIVE**
17	HP117	TV537	16169	NEGATIVE	NEGATIVE	NEGATIVE
18	HP118	TV450	640704	NEGATIVE	**POSITIVE**	NEGATIVE
19	HP119	TV293	132156	**POSITIVE**	**POSITIVE**	NEGATIVE
20	HP120	TV301	325537	NEGATIVE	NEGATIVE	NEGATIVE
21	HP121	TV281	104570	**POSITIVE**	**POSITIVE**	NEGATIVE
22	HP122	TV278	104950	NEGATIVE	NEGATIVE	NEGATIVE
23	HP123	TV613	492940	NEGATIVE	NEGATIVE	NEGATIVE
24	HP124	TV320	324955	NEGATIVE	NEGATIVE	NEGATIVE
25	HP125	TV183	161282	NEGATIVE	**POSITIVE**	**POSITIVE**
26	HP126	TV318	325054	NEGATIVE	NEGATIVE	**POSITIVE**
27	HP128	TV232	118830	NEGATIVE	**POSITIVE**	**POSITIVE**
28	HP129	TV311	323478	NEGATIVE	NEGATIVE	NEGATIVE
29	HP130	TV276	104968	**POSITIVE**	**POSITIVE**	**POSITIVE**
30	HP131	TV336	432683	NEGATIVE	NEGATIVE	NEGATIVE
31	HP133	TV418	534468	NEGATIVE	NEGATIVE	NEGATIVE
32	HP134	TV100	200907	**POSITIVE**	**POSITIVE**	**POSITIVE**
33	HP135	TV339	450561	NEGATIVE	NEGATIVE	NEGATIVE
34	HP136	TV163	310936	**POSITIVE**	**POSITIVE**	NEGATIVE
35	HP137	TV289	110929	NEGATIVE	NEGATIVE	NEGATIVE
36	HP138	TV632	300902	NEGATIVE	NEGATIVE	NEGATIVE
37	HP139	TV291	104404	NEGATIVE	NEGATIVE	NEGATIVE
38	HP140	TV426	534594	**POSITIVE**	**POSITIVE**	NEGATIVE
39	HP141	TV275	104968	**POSITIVE**	**POSITIVE**	NEGATIVE
40	HP142	TV346	432704	NEGATIVE	NEGATIVE	NEGATIVE
41	HP143	TV315	325114	NEGATIVE	NEGATIVE	NEGATIVE
42	HP144	TV529	373797	NEGATIVE	NEGATIVE	NEGATIVE
43	HP145	TV354	474152	**POSITIVE**	**POSITIVE**	**POSITIVE**
44	HP147	TV312	325108	NEGATIVE	NEGATIVE	NEGATIVE
45	HP148	TV499	352322	**POSITIVE**	**POSITIVE**	**POSITIVE**
46	HP149	TV612	481471	NEGATIVE	NEGATIVE	NEGATIVE
47	HP150	TV531	461598	NEGATIVE	NEGATIVE	NEGATIVE

48	HP151	TV51	324859	**POSITIVE**	**POSITIVE**	**POSITIVE**
49	HP152	TV337	473547	NEGATIVE	NEGATIVE	NEGATIVE
50	HP153	TV345	473680	NEGATIVE	NEGATIVE	NEGATIVE
51	HP155	TV530	102505	NEGATIVE	NEGATIVE	NEGATIVE
52	HP156	TV99	200907	**POSITIVE**	**POSITIVE**	**POSITIVE**
53	HP157	TV321	333639	NEGATIVE	NEGATIVE	NEGATIVE
54	HP158	TV609	482167	NEGATIVE	NEGATIVE	NEGATIVE
55	HP159	TV414	531390	NEGATIVE	NEGATIVE	NEGATIVE
56	HP161	TV390	811545	NEGATIVE	NEGATIVE	NEGATIVE
57	HP163	TV1X	392523	**POSITIVE**	**POSITIVE**	NEGATIVE
58	HP164	TV325	324297	NEGATIVE	NEGATIVE	NEGATIVE
59	HP165	TV460	599575	**POSITIVE**	**POSITIVE**	**POSITIVE**
60	HP166	TV243	640429	**POSITIVE**	**POSITIVE**	**POSITIVE**
61	HP168	TV272	110972	NEGATIVE	NEGATIVE	NEGATIVE
62	HP169	TV346	432702	NEGATIVE	NEGATIVE	NEGATIVE
63	HP171	TV235	650459	NEGATIVE	NEGATIVE	NEGATIVE
64	HP172	TV646	313492	NEGATIVE	**POSITIVE**	NEGATIVE
65	HP173	TV385	811398	**POSITIVE**	**POSITIVE**	NEGATIVE
66	HP174	TV502	352314	**POSITIVE**	**POSITIVE**	NEGATIVE
67	HP175	TV493	340200	**POSITIVE**	**POSITIVE**	NEGATIVE
68	HP176	TV252	132531	NEGATIVE	NEGATIVE	NEGATIVE
69	HP177	TV305	333432	NEGATIVE	NEGATIVE	NEGATIVE
70	HP178	TV541	751338	NEGATIVE	**POSITIVE**	NEGATIVE

MASTER CHART OF CULTURE BLINDED (PCR ASSAY DONE USING HOT STAR Taq mix)

Serial no	Code no	Sample no	Hospital no	Wet mount	culture	PCR2
1	HP101	TV258	132526	**POSITIVE**	**POSITIVE**	**POSITIVE**
2	HP102	TV270	431497	NEGATIVE	NEGATIVE	NEGATIVE
3	HP103	TV267	110668	NEGATIVE	NEGATIVE	NEGATIVE
4	HP104	TV395	103192	NEGATIVE	NEGATIVE	NEGATIVE
5	HP105	TV245	191152	NEGATIVE	NEGATIVE	NEGATIVE
6	HP106	TV181	212504	**POSITIVE**	**POSITIVE**	**POSITIVE**
7	HP107	TV304	325662	**POSITIVE**	**POSITIVE**	**POSITIVE**
8	HP108	TV650	357315	**POSITIVE**	**POSITIVE**	**POSITIVE**
9	HP109	TV333	473677	**POSITIVE**	**POSITIVE**	**POSITIVE**
10	HP110	TV6	404986	NEGATIVE	NEGATIVE	NEGATIVE
11	HP111	TV341	461274	NEGATIVE	NEGATIVE	NEGATIVE
12	HP112	TV335	440078	**POSITIVE**	**POSITIVE**	**POSITIVE**
13	HP113	TV306	333524	NEGATIVE	NEGATIVE	NEGATIVE
14	HP114	TV303	325776	**POSITIVE**	**POSITIVE**	**POSITIVE**
15	HP115	TV302	325444	NEGATIVE	NEGATIVE	NEGATIVE
16	HP116	TV550	263071	**POSITIVE**	**POSITIVE**	**POSITIVE**
17	HP117	TV537	16169	NEGATIVE	NEGATIVE	NEGATIVE
18	HP118	TV450	640704	NEGATIVE	**POSITIVE**	**POSITIVE**
19	HP119	TV293	132156	**POSITIVE**	**POSITIVE**	NEGATIVE
20	HP120	TV301	325537	NEGATIVE	NEGATIVE	NEGATIVE
21	HP121	TV281	104570	**POSITIVE**	**POSITIVE**	**POSITIVE**
22	HP122	TV278	104950	NEGATIVE	NEGATIVE	NEGATIVE
23	HP123	TV613	492940	NEGATIVE	NEGATIVE	NEGATIVE
24	HP124	TV320	324955	NEGATIVE	NEGATIVE	NEGATIVE
25	HP125	TV183	161282	NEGATIVE	**POSITIVE**	**POSITIVE**
26	HP126	TV318	325054	NEGATIVE	NEGATIVE	NEGATIVE
27	HP128	TV232	118830	NEGATIVE	**POSITIVE**	**POSITIVE**
28	HP129	TV311	323478	NEGATIVE	NEGATIVE	NEGATIVE
29	HP130	TV276	104968	**POSITIVE**	**POSITIVE**	**POSITIVE**
30	HP131	TV336	432683	NEGATIVE	**NEGATIVE**	**NEGATIVE**
31	HP133	TV418	534468	NEGATIVE	NEGATIVE	NEGATIVE
32	HP134	TV100	200907	**POSITIVE**	**POSITIVE**	**POSITIVE**
33	HP135	TV339	450561	NEGATIVE	NEGATIVE	NEGATIVE
34	HP136	TV163	310936	**POSITIVE**	**POSITIVE**	**POSITIVE**
35	HP137	TV289	110929	NEGATIVE	NEGATIVE	NEGATIVE
36	HP138	TV632	300902	NEGATIVE	NEGATIVE	NEGATIVE
37	HP139	TV291	104404	NEGATIVE	NEGATIVE	NEGATIVE
38	HP140	TV426	534594	**POSITIVE**	**POSITIVE**	**POSITIVE**
39	HP141	TV275	104968	NEGATIVE	**POSITIVE**	**POSITIVE**
40	HP142	TV346	432704	NEGATIVE	NEGATIVE	NEGATIVE
41	HP143	TV315	325114	NEGATIVE	NEGATIVE	NEGATIVE
42	HP144	TV529	373797	NEGATIVE	NEGATIVE	NEGATIVE
43	HP145	TV354	474152	**POSITIVE**	**POSITIVE**	**POSITIVE**
44	HP147	TV312	325108	NEGATIVE	NEGATIVE	NEGATIVE
45	HP148	TV499	352322	**POSITIVE**	**POSITIVE**	**POSITIVE**
46	HP149	TV612	481471	NEGATIVE	NEGATIVE	NEGATIVE
47	HP150	TV531	461598	NEGATIVE	NEGATIVE	NEGATIVE

48	HP151	TV51	324859	**POSITIVE**	**POSITIVE**	**POSITIVE**
49	HP152	TV337	473547	NEGATIVE	NEGATIVE	**POSITIVE**
50	HP153	TV345	473680	NEGATIVE	NEGATIVE	NEGATIVE
51	HP155	TV530	102505	NEGATIVE	NEGATIVE	NEGATIVE
52	HP156	TV99	200907	**POSITIVE**	**POSITIVE**	**POSITIVE**
53	HP157	TV321	333639	NEGATIVE	NEGATIVE	NEGATIVE
54	HP158	TV609	482167	NEGATIVE	NEGATIVE	NEGATIVE
55	HP159	TV414	531390	NEGATIVE	NEGATIVE	NEGATIVE
56	HP161	TV390	811545	NEGATIVE	NEGATIVE	NEGATIVE
57	HP163	TV1X	392523	**POSITIVE**	**POSITIVE**	**POSITIVE**
58	HP164	TV325	324297	NEGATIVE	NEGATIVE	NEGATIVE
59	HP165	TV460	599575	**POSITIVE**	**POSITIVE**	**POSITIVE**
60	HP166	TV243	640429	**POSITIVE**	**POSITIVE**	**POSITIVE**
61	HP168	TV272	110972	NEGATIVE	NEGATIVE	NEGATIVE
62	HP169	TV346	432702	NEGATIVE	NEGATIVE	NEGATIVE
63	HP171	TV235	650459	NEGATIVE	NEGATIVE	NEGATIVE
64	HP172	TV646	313492	NEGATIVE	**POSITIVE**	**POSITIVE**
65	HP173	TV385	811398	**POSITIVE**	**POSITIVE**	**POSITIVE**
66	HP174	TV502	352314	**POSITIVE**	**POSITIVE**	**POSITIVE**
67	HP175	TV493	340200	**POSITIVE**	**POSITIVE**	**POSITIVE**
68	HP176	TV252	132531	NEGATIVE	NEGATIVE	NEGATIVE
69	HP177	TV305	333432	NEGATIVE	NEGATIVE	NEGATIVE
70	HP178	TV541	751338	NEGATIVE	**POSITIVE**	**POSITIVE**

Sl.No	TV No.	Hosp No.	Age	Wet Mount	Culture	PCR	Address	Sympy/ Asymp	occu	on Rx	HAART	HIV	HbsAg	HCV Ab	VDRL
1	TV1	838385C	33yrs	negative	negative	negative	vellore	1	hw		1	1	1	0	1
2	TV2	519964C	25yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
3	TV3	433489C	25yrs	negative	negative	negative	kadapa	1	hw		1	1	1	1	1
4	TV4	837059C	22yrs	negative	negative	negative	Thiruvarur	2	hw		1	1	0	0	1
5	TV5	833514C	48yrs	negative	negative	negative	vellore	2	hw		1	1	1	1	1
6	TV6	842658c	52yrs	negative	negative	negative	W.Bengal	2	hw		1	1	0	0	0
7	TV7	844975C	35yrs	negative	negative	negative	jharkhand	2	hw		1	1	0	0	0
8	TV8	812640C	30yrs	negative	negative	negative	chittor	1	hw		1	1	0	0	0
9	TV9	839471C	31yrs	negative	negative	negative	vellore	1	hw		2	1	0	0	0
10	tv10	852377C	30yrs	negative	negative	negative	vellore	2	hw		1	1	1	0	1
12	TV12	816504c	22yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
13	TV13	210964C	38yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	1
14	TV14	32964B	35yrs	negative	negative	negative	Kanamang	1	hw		1	1	0	0	0
15	TV15	185743C	36yrs	negative	negative	negative	Pochampaly	1	hw		1	1	1	0	1
18	TV18	865439c	31yrs	negative	negative	negative	chittor	2	hw		1	1	1	1	1
19	TV19	477546c	36yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	0
20	TV20	066498b	29yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
21	TV21	714471c	49yrs	negative	negative	negative	Namakal	1	hw		1	1	0	0	0
22	TV22	862739c	34yrs	negative	negative	negative	jharkhand	1	acct		1	1	0	1	2
23	TV23	806080C	34yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
24	TV24	401173c	30yrs	**positive**	**positive**	negative	chittor	1	hw		1	1	0	0	0
25	TV25	234502c	25yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
26	TV26	705186c	33yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
27	TV27	877008c	52yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	1
28	TV28	875767c	52yrs	negative	negative	negative	jharkhand	1	hw		1	1	0	0	1
29	TV29	872818c	34yrs	negative	negative	negative	krishnagiri	2	hw		1	1	0	0	1
30	TV30	893737c	28yrs	negative	negative	negative	jharkhand					**2**	0	0	0
31	TV31	897306c	27yrs	negative	negative	negative	vellore	2	hw		1	1	0	0	0
32	TV32	987228c	24yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	1
33	TV33	842117C	39yrs	negative	negative	negative	Karanatak	2	hw		1	1	2	1	0
34	TV34	869699c	32yrs	negative	negative	negative	vellore	2	hw		1	1	1	1	1
35	TV35	373438c	45yrs	negative	negative	negative	vellore	2	hw		1	1	0	0	0
36	TV36	503684c	40yrs	negative	negative	negative	chittor	1	hw		2	1	1	1	0
37	TV37	872424c	47yrs	negative	negative	negative	chittor	1	labourer		1	1	0	0	1
38	tv38	146742c	30yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	1

39	tv39	466371c	39yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	0
40	tv40	649355a	29yrs	negative	negative	negative	Arcot	1	hw		2	1	0	0	0
41	tv41	908791c	27yrs	negative	negative	negative	krishnagiri					**2**	0	0	0
42	tv42	022611c	49yrs	negative	negative	negative	vellore	1	hw		2	1	0	0	0
43	tv43	201481c	31yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
44	tv44	910616c	34yrs	negative	negative	negative	W Bengal	2	hw		1	1	1	1	1
45	tv45	309215c	29yrs	negative	negative	negative	Tirupattur	2	hw		1	1	1	0	1
46	TV46	782981b	34yrs	negative	negative	negative	vellore	2	hw		1	1	0	0	0
47	tv47	919802c	35yrs	negative	negative	negative	cedllore	1	hw		1	1	1	1	0
48	TV48	919974c	37yrs	negative	negative	negative	chittor	1	hw		1	1	0	0	0
49	TV49	922019c	36yrs	negative	negative	negative	chittor	2	hw		1	1	0	0	1
50	TV50	899317C	27yrs	negative	negative	negative	Kadapa	2	hw		1	1	0	0	1
51	TV51	919915c	30yrs	negative	negative	negative	jharkhand	2	hw		1	1	0	0	1
52	TV52	234838b	40yrs	negative	negative	negative	chittor	1	hw		1	1	1	0	1
53	TV53	911587c	26yrs	negative	negative	negative	vellore	1	coolie		1	1	1	1	2
54	TV54	847794c	30yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
55	TV55	900810c	27yrs	negative	negative	NO SWAE	chittor								
56	TV56	943139c	29yrs	negative	negative	NO SWAE	vellore								
57	TV57	713510c	29yrs	negative	negative	negative	Tirupattur	1	hw		2	1	0	0	1
58	TV58	594849c	35yrs	negative	negative	negative	vellore	2	hw	1	1	0	0	0	0
59	TV59	964349c	23yrs	negative	negative	negative	vellore	2	hw		1	1	0	0	1
60	TV60	691341c	38yrs	negative	negative	negative	vellore	2	HW		1	1	0	0	0
61	TV61	963229a	33yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	0
63	tv63	728242a	37yrs	negative	negative	negative	Gudiyatham	2	hw		1	1	1	0	0
64	TV64	163989C	31yrs	negative	negative	negative	Kovai	2	hw		1	1	1	0	1
65	TV65	952587c	48yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	2
66	TV66	398540x	24yrs	negative	negative	negative	chad	1	hw		1	1	0	0	0
67	TV67	267515x	38yrs	negative	negative	negative	chad	1	hw		1	1	0	0	0
69	tv69	972070c	29yrs	negative	negative	negative	chittor	1	hw		1	1	1	0	1
70	tv70	405073x	52yrs	negative	negative	negative	chad	1	hw		1	1	0	0	0
71	tv71	393074x	32yrs	negative	negative	negative	chad	1	hw		1	1	0	0	0
72	tv72	116931x	23yrs	negative	negative	negative	chad	1	hw		1	1	0	1	0
73	tv73	415965x	35yrs	negative	negative	**positive**	chad	1	hw		1	1	0	1	0
74	tv74	197684C	32yrs	negative	negative	negative	Villupurum	2	hw		1	1	0	0	0
75	TV75	561449a	37yrs	negative	negative	negative	vellore	2	hw		1	1	0	0	0
76	tv76	502921c	33yrs	negative	negative	**positive**	A.P	1	hw		2	1	0	1	0

77	tv77	806080C	34yrs	negative	negative	negative	vellore	2	coolie	1	1	1	1	1
78	TV78	972680c	41yrs	negative	negative	negative	chittor	1	hw	1	1	0	0	0
79	tv79	333331C	26yrs	negative	negative	negative	Arcot	1	coolie	1	1	1	0	1
80	TV80	226342c	35yrs	negative	negative	negative	vellore	1	hw	1	1	0	1	0
82	tv82	973774c	34yrs	negative	negative	negative	jharkhand	2	hw	1	1	0	0	0
83	tv83	349511x	33yrs	negative	negative	negative	chad	2	hw	1	1	0	1	0
84	tv84	292639x	28yrs	negative	negative	negative	chad	1	hw	1	1	0	1	0
85	tv85	340918x	26yrs	negative	negative	negative	vellore	1	hw	1	1	0	1	0
86	tv86	395913x	27yrs	negative	negative	negative	chad	1	hw	1	1	0	1	0
87	tv87	977632c	29yrs	negative	negative	negative	W.Bengal	1	hw	1	1	0	0	0
88	tv88	929119c	21y	negative	negative	negative	chittor	2	hw	1	1	1	1	1
89	tv89	972071c	31yrs	negative	negative	negative	vellore	1	hw	1	1	0	0	0
90	tv90	163982c	29yrs	negative	negative	negative	vellore	1	hw	2	1	1	1	1
91	tv91	974780C	28yrs	negative	negative	negative	chittor	1	hw	1	1	1	1	0
92	TV92	955826B	45y	negative	negative	negative	Gudiyatham	1	hw	1	1	0	0	1
93	TV93	242460c	34yrs	negative	negative	negative	Cuddapha	1	hw	2	1	0	1	2
94	TV94	979123C	26yrs	negative	negative	negative	T.Mali	1	hw	1	1	1	1	1
95	TV95	427555c	32yrs	negative	negative	negative	Kadapa	1	hw	1	1	1	1	1
193	TV193	13226D	27yrs	negative	negative	negative	Thhirupattur	1	hw	1	1	1	1	0
194	TV194	130341D	39yrs	negative	negative	negative	vellore	2	hw	1	1	1	1	2
195	TV195	381888X	23yrs	negative	negative	negative	chad	1	hw	1	1	0	0	0
196	TV196	390539X	37yrs	negative	negative	negative	chad	1	hw	1	1	0	0	0
197	TV197	138151D	35yrs	negative	negative	negative	vellore	1	hw	1	1	0	0	1
198	TV198	123459D	36yrs	negative	negative	negative	vellore	1	hw	1	1	1	1	1
199	TV199	096479D	23yrs	negative	negative	negative	krishnagiri	2	hw	1	1	1	0	1
200	TV200	123019D	33yrs	negative	**positive**	**positive**	vellore	2	hw	1	1	1	1	1
201	TV201	137097D	27yrs	negative	negative	negative	krishnagiri			1	1	0	0	1
202	TV202	675139C	22yrs	negative	negative	negative	vellore	1	hw	1	1	1	0	1
203	TV203	379175X	25yrs	negative	negative	negative	chad	2	hw	1	1	0	0	0
204	TV204	366360X	25yrs	negative	**positive**	**positive**	chad	1	hw	1	1	0	0	0
205	TV205	355419X	36yrs	negative	negative	negative	chad	1	hw	1	1	0	0	0
206	TV206	407743X	38yrs	negative	negative	negative	chad	1	hw	1	1	0	1	0
207	TV207	412225X	28yrs	negative	negative	negative	chad	1	hw	1	1	0	1	0
208	TV208	380998X	27yrs	negative	negative	negative	chad	2	hw	1	1	0	1	0
209	TV209	971794C	23yrs	negative	negative	negative	vellore	2	hw	1	1	0	0	1
210	TV210	827618C	27yrs	negative	negative	negative	W.Bengal	1	hw	1	1	1	1	1

211	TV211	395913X	27yrs	negative	negative	negative	chad	1	hw			1	0	1	0
212	TV212	410113X	26yrs	negative	negative	negative	chad	1	hw		1	1	0	1	0
213	TV213	272525X	32yrs	negative	negative	negative	chad	1	hw		1	1	0	1	0
214	TV214	167952D	27yrs	negative	negative	**positive**	vellore	1	hw		1	1	1	0	2
215	TV215	173850B	30yrs	negative	negative	negative	chittor	1	hw		1	1	0	0	0
216	TV216	430546X	39yrs	negative	negative	negative	chad	2	hw		1	1	0	1	0
217	TV217	214893X	32yrs	negative	negative	negative	chad	2	hw		1	1	0	0	0
218	TV218	176358D	30yrs	negative	negative	negative	kadapa	1	teacher		1	1	1	1	1
219	TV219	165801D	36yrs	negative	negative	negative	Cuddapha	1	hw		1	1	1	1	1
220	TV220	148799D	35yrs	negative	negative	negative	Chittor	1	hw		1	1	1	1	1
221	TV221	276955X	33yrs	negative	negative	negative	chad	1	hw		1	1	0	0	0
222	TV222	671767C	38yrs	negative	negative	negative	Chittor	1	hw		1	1	1	1	1
223	TV223	180923D	50yrs	negative	negative	negative	t.v mali	1	labourer		1	1	1	1	1
224	TV224	346514X	25yrs	negative	negative	negative	chad	2	hw		1	1	0	1	0
225	TV225	116931X	23yrs	negative	negative	negative	chad	1	hw		1	1	0	1	0
226	TV226	194360C	30yrs	negative	CULTURE	MEDIA SP	vellore								
227	TV227	185248D	32yrs	negative	CULTURE	MEDIA SP	vellore								
228	TV228	332759X	38yrs	negative	negative	negative	chhad	1	hw		1	1	0	1	0
229	TV229	426122X	31yrs	negative	negative	negative	Chittor	1	hw		1	1	0	1	0
230	TV230	896338B	33yrs	negative	negative	**positive**	vellore	1	hw		1	1	0	0	1
231	TV231	113983D	36yrs	negative	negative	negative	krishnagiri	2	hw		1	1	1	1	1
232	TV232	118830D	48yrs	negative	**positive**	**positive**	Erode	1	labourer		1	1	1	1	1
233	TV233	183618D	38yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
234	TV234	037608D	24yrs	negative	negative	negative	jharkhand	1	service		1	1	0	0	0
235	TV235	984485C	38yrs	negative	negative	negative	vellore	2	hw		2	1	1	1	1
236	TV236	207188D	25yrs	negative	negative	negative	Arrakonam	1	hw		1	1	1	1	0
237	TV237	218478D	30yrs	negative	negative	negative	W.Bengal	1	hw		1	1	1	1	1
238	TV238	201209B	38yrs	negative	negative	negative	Chittor	1	hw		1	1	1	1	0
239	TV239	184135D	31yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
240	TV240	147161C	40yrs	negative	negative	negative	vellore	1	hw		2	1	0	0	0
241	TV241	197809D	46yrs	negative	negative	negative	banglore	1	hw		1	1	1	1	1
242	TV242	175606D	28yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
243	TV243	042930D	27yrs	negative	negative	negative	Arcot	2	hw		1	1	1	1	1
244	TV244	791462C	29yrs	negative	negative	negative	vellore	1	hw		1	1	1	0	1
245	TV245	219429D	22yrs	negative	negative	negative	krishnagiri	2	hw		1	1	1	1	1
246	TV246	025607C	26yrs	negative	negative	negative	Cheriyar	1	hw		1	1	1	0	1

247	TV247	963229A	33yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
248	TV248	206152D	33yrs	negative	negative	negative	west beng	2	hw		1	1	1	1	1
249	TV249	199433D	31yrs	negative	negative	negative	chittor	2	hw		1	1	1	0	1
250	TV250	230924D	50yrs	negative	negative	negative	Tanjore	1	hw		1	1	1	1	1
251	TV251	842264C	51y	negative	negative	negative	vellore	2	hw		1	1	1	1	1
252	TV252	005441D	32yrs	negative	negative	negative	W.Bengal	2	hw		1	1	1	1	1
253	TV253	109891D	46yrs	negative	negative	negative	chittor	1	hw		2	1	1	1	1
254	TV254	054988D	30yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
255	TV255	235476D	27yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
256	TV256	609510C	38yrs	negative	negative	negative	vellore	1	hw		1	1	0	1	0
257	TV257	225038C	28yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
258	TV258	234973D	25yrs	negative	**positive**	**positive**	Apradesh	1	hw		1	1	1	1	1
259	TV259	235155D	36yrs	negative	negative	negative	Pondi	1	hw		1	1	1	0	0
260	TV260	239479D	44y	negative	negative	negative	vellore	1	hw		1	1	1	2	1
261	TV261	234128D	36yrs	negative	negative	negative	Trichy	1	hw		1	1	1	1	1
262	TV262	121508C	36yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
263	TV263	234002D	35yrs	negative	negative	**positive**	W.Bengal	1	hw		1	1	1	1	1
264	TV264	225152D	36yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
265	TV265	800576D	39yrs	negative	negative	negative	vellore	2	hw		1	1	0	0	0
266	TV266	171143D	36yrs	negative	negative	negative	T.Mali	1	hw		1	1	1	0	1
267	TV267	941641D	34yrs	negative	negative	negative	wBengal	1	hw		1	1	0	0	0
268	TV268	837931C	32yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
269	TV269	243839D	21y	negative	negative	negative	Villupurum	1	coolie		1	1	1	1	1
270	TV270	246815D	47yrs	negative	negative	negative	A.P	1	hw		1	1	1	1	1
271	TV271	248593D	28yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
272	TV272	809616C	36yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
273	TV273	248593D	36yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
274	TV274	246668D	36yrs	negative	negative	negative	A.P	1	hw		1	1	1	1	1
275	TV275	159625D	36yrs	negative	negative	negative	krishnagiri	1	hw		1	1	1	1	1
276	TV276	992888C	28yrs	negative	negative	negative	vellore	2	hw		1	1	1	0	1
277	TV277	252357D	46yrs	negative	negative	negative	krishnagiri	1	farmer		1	1	1	1	1
278	TV278	248548D	45yrs	negative	negative	negative	kadapa	1	hw		1	1	1	1	2
279	TV279	254014D	36yrs	negative	negative	negative	wBengal	1	hw		1	1	1	1	1
280	TV280	187575C	45yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
281	TV281	913971C	52yrs	negative	negative	negative	Chittor	1	hw		1	1	1	1	1
282	TV282	633679C	32yrs	negative	negative	negative	krishnagiri	1	hw		1	1	1	1	0

283	TV283	255861D	25yrs	negative	negative	negative	t.v mali	1	hw		1	1	0	0	0
284	TV284	168771C	26yrs	negative	negative	negative	Chittor	1	hw		1	1	1	0	1
285	TV285	283910C	30yrs	negative	negative	negative	Chittor	1	labourer		1	1	0	0	0
286	TV286	114496D	28yrs	negative	**positive**	**positive**	vellore	2	hw		1	1	1	1	1
287	TV287	260190D	50yrs	negative	negative	negative	chittor	1	hw		1	1	1	1	1
288	TV288	058837D	39yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
289	TV289	866807C	22yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
290	TV290	042254D	37yrs	negative	negative	negative	Erode	2	labourer		1	1	1	1	0
291	tv291	802700c	41yrs	negative	negative	negative	Chittor	1	hw		1	1	1	1	1
292	TV292	206090D	32yrs	negative	negative	negative	Kanchiuram	1	hw		1	1	1	1	1
293	tv293	096479D	23yrs	negative	negative	negative	krishnagiri	2	hw		1	1	1	0	1
294	tv294	885619D	36yrs	negative	negative	negative	Salem	1	hw		1	1	0	0	0
295	tv295	265239D	38yrs	negative	negative	negative	Chittor	1	hw		1	1	1	1	1
296	TV296	264943c	28yrs	negative	negative	negative	vellore	1	labourer		1	1	1	1	1
297	TV 297	736882c	32yrs	negative	negative	negative	vellore	1	labourer		1	1	1	1	1
298	tv298	264943c	28yrs	negative	negative	negative	vellore	1	hw		1	1	0	0	0
299	tv299	266974D	26yrs	negative	negative	negative	Vellore	1	Chital		1	1	0	1	1
300	TV300	984485C	38yrs	negative	negative	negative	vellore	2	hw	1	1	1	1	1	1
301	TV301	149948D	40yrs	negative	negative	negative	jharkhand	1	hw		1	1	1	1	1
302	tv302	268623D	26yrs	negative	negative	negative	CHITTOR	1	HW		1	1	1	0	1
303	TV303	271338D	27yrs	negative	negative	negative	Madurai	2	HW		1	1	1	1	1
304	TV304	002975D	37yrs	negative	negative	negative	vellore	1	hw		1	1	1	1	1
305	TV305	978195c	35yrs	negative	negative	negative	vellore	2	labourer		1	1	0	0	1
306	tv306	267087D	30yrs	negative	negative	**positive**	Krishnagiri	2	HW	0	1	1	1	1	1
307	TV307	186988D	33yrs	negative	negative	negative	vellore	1	coolie		1	1	1	1	1