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# Evaluation of pcr in the molecular diagnosis of trichomonas vaginalis infection in comparison with other conventional methods

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**Abstract**

*Trichomonas vaginalis* (*T. vaginalis*) is a common pathogen with worldwide distribution. It is estimated that worldwide 180 million people are infected annually. Trichomoniasis is associated with vaginitis, cervicitis, low birth weight, and preterm delivery. PCR has the advantage of high sensitivity, shorter time for diagnosis and the ability to detect nonviable or defective organism. In this study we used these three methods for evaluation of PCR in comparison with conventional methods like wet mount and culture in the detection of *T. vaginalis* in vaginal discharge. Three vaginal swab specimens were obtained from each of 200 cases, of the age group 18-40years, both symptomatic and asymptomatic females attending Gynaecology OPD(50) and Family planning OPD(50) at Gandhi hospital, Secunderabadand two FSW(Female sex workers) clinics (100) in highly concentrated areas of them in Hyderabad, for validation of various forms of Trichomonas vaginalis diagnostic procedures. One swab was immediately examined by wetmount microscopy, a second swab was placed in Wittington's medium for cultivation, and other swab is placed in 2SP transport medium for PCR for *T.vaginalis*. A total of 58 samples positive in one or more tests were identified: 11 (5.5%) infections were detected by wet mount microscopy, and 30 (15%) positives in culture respectively. PCR was positive in 50 (25%) samples. PCR appears to be the most sensitive method with high detection rate and method of choice for detection of genital infections with *T. vaginalis*.

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**Introduction**

Worldwide, *Trichomonas vaginalis* causes approximately 180 million new infections per year, making it the most prevalent non-viral sexually transmitted disease (STD) agent. (Petrin *et al*., 1998; Kengue *et al*., 1994; Madico *et al*., 1998).

Infections in women can cause vaginitis, urethritis, and cervicitis, and complications include premature labor, lowbirth-weight offspring, and post abortion or post hysterectomy infection (Shaio, *et al*., 1997). It has been estimated that 10 to 50% of *T. vaginalis* infections in women are asymptomatic and in men the proportion may even be higher. (Burstein, G. R *et al*., 1999) This parasite has also been implicated as a cofactor in the transmission of the human immunodeficiency virus and other nonulcerative STD agents. However, since the incidence of *T. vaginalis* infection is highest for groups with a high prevalence of other STDs, this latter hypothesis remains to be confirmed (Madico *et al*., 1998). In addition, a relationship between *T. vaginalis* infec-tion and cervical cancer has recently been suggested (Zhang *et al*., 1995).

The most common tool for diagnosis of *T. vaginalis* infection is still microscopic examination of wet mount preparations, which has a sensitivity of approximately 38-60%. (Fouts *et al*., 1980) Microscopic examination of cultures of the parasite in specialized mediaimproves the sensitivity to 70-85% (Gelbart *et al*., 1990; Schmid *et al.,* 1989). However, the quality of these diagnostic tests is strongly dependent on theskills and experience of the microscopist and also on the quality of the sample. Diagnosis traditionally depends on the microscopic observation of motile protozoa in vaginal discharge. Culture requires a special medium and the result takes up to 7 days. Diagnostic improvements have been suggested since years. Finally, molecular techniques such as fluorescent in situ hybridization, oligonucleotide probing, and PCR have been developed. (Petrin *et al*., 1998; Kengue *et al*., 1994; Zhang *et al*., 1995; Muresu *et al*., 1994; Philips Heine *et al* 1997; Riley *et al.,* 1992; Rubino *et al.,* 1991).

To date, numerous *T. vaginalis* specific PCR assays have been described. Examples of targets include the ferridoxin gene, beta tubulin gene, highly repeated DNA sequencing and 18s ribosomal genes. (Riley *et al.,* 1992; Kengue *et al.,* 1994; Madico *et al.,* 1998; Mayta *et al.,* 2000).

Marcia M. Hobbs *et al*., compared culture and PCR ELISA in urethral swabs, urine and semen for TV detection in male sexual partners of women with trichomoniasis identified by wet mount and culture. TV was detected more often in men with wet mount positive partners emphasizing the importance of partner evaluation and treatment. Even with a sensitive PCR assay, reliable detection of TV in male partners required multiple specimens.

Charlotte Gaydos *et al*., (2006), compared Gene Probe transcription mediated amplification TV research assay and real time PCR for TV detection using a Roche Light Cycler instrument with female self-obtained vaginal swab samples and male urine samples. The Gen Probe TMA assay is commercially available as an analyte specific reagent and offers laboratories a highly sensitive and specific assay for use clinically.

Rasoul Jamali *et al*., (2006), compared diagnosis of Trichomonas vaginalis infection using PCR method to culture and wet mount microscopy and concluded that PCR had high sensitivity and slightly less specificity than wet mount taking culture as the standard.

This study was done to diagnose both symptomatic and asymptomatic female patients with Trichomonas vaginalis infection by Microscopy, Culture and PCR and to compare the efficacy of each of the above diagnostic modalities in terms of rapidity, sensitivity and specificity.

**Material and methods**

**Sample size**: A total of 200 females, of the age group 18-40 years, both symptomatic and asymptomatic females attending Gynaecology OPD (50) and Family planning (FP) OPD (50) at Gandhi hospital, Secunderabad and two FSW (Female sex workers) clinics (100) in highly concentrated areas of them in Hyderabad from November 2008 to February 2010.

Relevant data from the subjects are recorded. All the subjects were informed about the study and they agreed to participate in the study.

*Specimen collection*

Females with complaints of vaginal discharge, itching, dysuria and dyspareunia were considered as symptomatic and those without symptoms of trichomoniasis were considered as asymptomatic in the study. At the time of per-speculum examination three vaginal swabs from the posterior fornix and also touching both lateral fornices and middle third of vaginal wall were taken, using sterile Dacron swabs. Specimens collected prior to disinfection or local antibiotic used for routine microscopic examination, the second one was used to inoculate the culture medium and third swab was placed in 0.5ml of 2SP transport medium and stored at -20$^{o}$C.

*Wet mount preparation*

The swab inoculated with vaginal discharge for each patient was gently agitated in one drop of normal saline on a clean slide and then covered with a cover slip. The wet mount was examined with 40 objective and the presence of motile *T. vaginalis* was detected by the characteristic twitching motility (Fig.1).

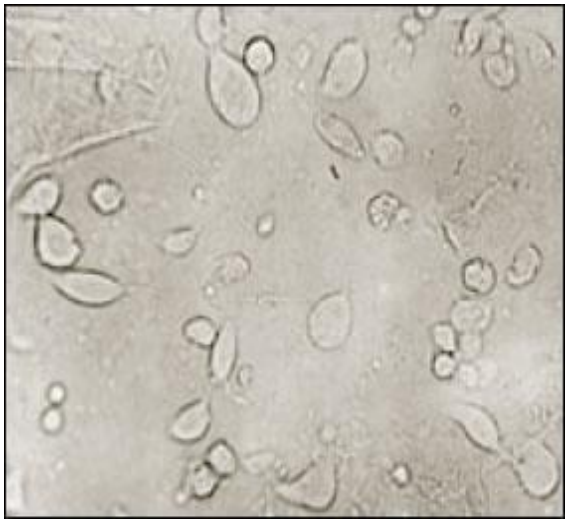

**Fig.1.** Wet mount of *T. Vaginalis, T. vaginalis culture*

To prepare Whittington medium, Trichomonas agar base is added to distilled water and sterilized by autoclave for 15min at 15lbs pressure (121$^{o}$C). It is cooled to 50$^{o}$C and aseptically horse serum is added.

To produce the axenic isolates the medium is supplemented with the antibiotics (Penicillin and Streptomycin). The culture medium was dispersed in a screw capped tubes in the volume of 5ml each and stored at 4$^{o}$C.

*Cultivation*

Before inoculation of medium, the culture tubes were warmed up to 37$^{o}$C for 15min. The vaginal swabs were placed into the medium and left to incubate at 37 $^{o}$C for 7days. The cultures were examined microscopically on days 2, 5 and 7 after inoculation. A positive result is defined as the presence of motile *T. Vaginalis* at any time, a negative result was defined as absence of motile *T. vaginalis* at all readings (Fig.2).

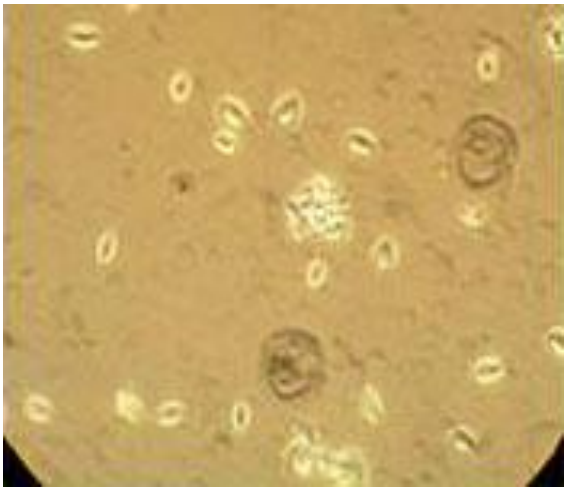

**Fig. 2.** Trichomonas in culture.

*Trichomonas vaginalis Polymerase chain reaction (TVPCR) (Rosche NG/CT kit modification)*

DNA Extraction: 2ml screw cap tube is centrifuged for 10min after transferring 250µl of sample suspension after vortexing thoroughly for 20 seconds. 250µl LYS is added to the supernatant and mixed by vortexing and incubated for 10min. 250µl DIL is added to each tube and mixed by vortexing and incubated for 10min. processed specimens are kept at room temperature for up to 2 hrs before transferring aliquots to the PCR reaction tubes.

PCR primers: The primers based on *T. vaginalis* β-tubulin gene for PCR identification were used. The sequences of primers were as follows:

β-tubulin 9/2 primer 1: Biotin- 5’ GCA TGT TGT GCC GGA CAT AAC CAT.

β-tubulin 9/2 primer 2: Biotin- 5’ CAT TGA TAA CGA AGC TCT TTA CGAT.

PCR protocol: PCR reactions were performed with an automated the rmocycler. The total volume of PCR reactions was 50µl of the master mix and 50µl of the DNA extracted were added to the PCR tube. The amplification was performed in the PCR tubes and the procedure is as follows:

Hold Program: 50°C- 2 min
Hold Program: 95°C- 5 min
CYCLE: Denaturation: 95°C- 45sec
Annealing: 62-52°C- 45sec 35 cycles
Amplification: 72°C- 60sec
Hold Program: 72°C- 7 min
Hold Program: 72°C- Not to exceed 24 hours

*Detection*

All reagents and samples were brought to room temperature. Working wash solution is prepared. 25µlof denatured amplicon is pippeted to micro well plate and incubated for 1hr at 37°C. After washing the plate 5 times AV-HRP and SUB-A were added as given in the insert of the ROCHE NG/CT kit. At last, stop solution is added and read under Elisa reader. Results were interpretated as shown in table 1.

**Table 1.** Interpretation of PCR results.

| Result $A_{450}$ | Interpretation |
|---|---|
| < 0.3 | DNA not detected. Negative. |
| ≥ 0.8 | DNA detected. Specimen is presumptive positive for *T. vaginalis*. |
| ≥ 0.3, < 0.8 | Equivocal. Results are inconclusive. Repeat PCR testing on specimen in duplicate. Two of three results over 0.5 shall report the specimen as positive. |

**Results**

*Clinical Examination*

Total vaginal swab specimens collected from women attending OPD and FSW clinics were examined. Females attending the family planning OPD were considered as control as they were asymptomatic at the time of presentation. During per speculum examination of patients, finding have shown that signs of trichomoniasis are present in 82(82%) of FSWs, 50(100%) of females attending the gynaecology OPD and in 23(46%) of the females attending the family planning OPD i.e., controls. The most prevalent clinical symptom was vaginal discharge. (Table 2) The majority were in the age group of 21-30 years.

**Table 2.** Symptom wise distribution among FSW, GYNAEC OPD AND FP.

| Symptoms | FSW | GYNAEC | FP |
|---|---|---|---|
| Vaginal discharge | 78(78%) | 50(100%) | 23(46%) |
| Pruritus | 02(2%) | 04(8%) | 02(4%) |
| Odour | 10(10%) | 08(16%) | 01(2%) |
| Ulcer | 01(1%) | (0%) | (0%) |
| Burning micturition | 12(12%) | 03(6%) | 01(2%) |

*Microscopic examination and culture*

The incidence of positivity for Trichomonas vaginalis by wet mount was high (10%) in the female sex workers than in females attending Gynaecology OPD (2%). Positivity for TV by wet mount was negative in all control cases.

Culture showed 27% positives for Trichomonas vaginalis in the high risk group i.e., female sex workers and 6% positives in the females attending the gynaecology OPD.

The growth in Whittington's medium was observed in 30 out of 200. Totally 30 were positive both by culture and wetmount examination. Of these 19 samples were not detected by direct microscopic examination but were positive by cultivation method, and all wetmount positives were also shown positive by culture. (Table3) Considering the culture as the gold standard, the sensitivity of direct microscopy was 37% and specificity was 81%.

**Table 3**. Comparison of Wet mount positivity with culture positivity of *T.vaginalis*.

| | culture positive (n=30) | culture negative (n=170) |
|---|---|---|
| wet mount positive (n=10) | 11 | 00 |
| wet mount negative (n=90) | 19 | 170 |

All the 30 patients shown positive by culture and microscopy were all symptomatic with vaginal discharge on correlating with the examination. All the controls (family planning cases) were negative both by culture and also by wetmount examination.

*PCR results*

PCR assay was performed on all the 200 samples. β-tubul in primers were used for PCR assay. Detection was made with ELISA reader and results interpreted.

*T. vaginalis* was detected in 50(25%) of 200 samples. PCR could detect 28 positives which could not be detected by culture. 8 cases which were positive by culture were not detected by PCR. This may be due to the absence of β-tubulin gene in the trichomonas which were detected by culture. (Table4). The sensitivity of PCR was 88.37% and specificity was 83.5% taking culture as the gold standard.

**Table 4**. Comparison of PCR with culture positivity of *T. vaginalis*.

| | culture positive (n=30) | culture negative (n=170) |
|---|---|---|
| PCR positive (n=50) | 22 | 28 |
| PCR negative (n=150) | 08 | 92 |

**Discussion**

Accurate diagnosis of Trichomoniasis in sexually active women is extremely important since *T. vaginalis* may be the cause of high morbidity and, in common with other non-ulcerative sexually transmitted diseases, may be regarded as a risk factor for contraction of HIV infection.

The most common tool for diagnosis of *T. vaginalis* infection is still microscopic examination of wet mount preparations and culture. Diagnostic improvements have been suggested in past years. Finally, molecular techniques such as probing and PCR have been developed. These molecular techniques could assist clinicians in achieving the correct diagnosis, leading to prompt, sensitive and specific treatment under syndromic management.

This study describes two prospective clinical studies performed to evaluate various diagnostic methods for the detection of Trichomoniasis in vaginal swab samples obtained from females.

In this study, most common affected age group was 21-30yrs (57.5%). This correlates well with other studies conducted by BM Agarwal *et al*. (2000) and Marcia M. Hobbs *et al*. (2006); Swygard *et al*. (2004).

Wet mount was positive in 10% females in the high risk group i.e., female sex workers and 2% in the study group attending Gynaecology OPD Similar observations were made in the studies conducted by Charles Beal *et al* (1992) 5.8%,Alex Van.

A Pillay *et al* (2004)- 6%, Angelika Stary *et al*. (2002)- 6.5%, Rasoul Jamali *et al*. (2006)-3.46%. The low positivity by wet mount may be due to delay in reaching the microscope after preparation of wet mount or due to low number of organisms.

Culture, positivity by Wittington medium for Trichomoniasis was 27% in female sex workers and 6% of females attending Gynaecology OPD and none in the control group attending Family Planning OPD. Studies supporting this result in females attending gynaecology OPD are-Charlotte Gaydos *et al*. (1997)- 6.6%, Alex Van Belkum *et al*. (1999)- 4.9-5.7%. Studies supporting culture positivity in high risk group (FSWs) as observed in this study are Khan *et al*. (1991)- 28.4%; Schwebke *et al*. (1999)- 26% and Adu Sarkode *et al*. (2004)- 27.5%.

The Molecular methods adopted for the detection of Trichomonas vaginalis in this study was Roche PCR which is the modification of the Roche PCR kit for NG, CT. According to T. Crucitti *et al* (2003) detection of Trichomonas vaginalis by the Roche NG, CT PCR kit is equal to other PCR kits for Trichomonias vaginalis detection. In this study, PCR was positive in 45% of the female sex workers. Other studies correlating well with the results are-Marcia M. Hobbs*et al* (2002) - 38.4%, Schwebke *et al*. (2002)- 52%.

In the study group attending Gynaecology, the PCR positivity was 10%, this compares well with the A.Pillay *et al* (2004)- 12.6% of females are positive by PCR for Trichomoniasis.

PCR was found to be superior among the three diagnostic modalities for the detection of Trichomonas vaginalis i.e, wet mount, culture and PCR and the incidence of Trichomoniasis is more in the female sex workers than females attending Gynaecology OPD and controls.

In this study, Sensitivity of Wet Mount was 37% & 33% and specificity 81% & 100%. Low sensitivity of the wet mount may due to the low viability of the organisms, low number of organisms etc. Other studies shown almost similar results are-Charlotte A. Gaydos *et al* (1998)- 36% sensitive and 99% specific.

Sensitivity of the PCR in this study was 70-100% and specificity 64-95%. Studies correlating with this finding are- Marcia M. Hobbs *et al*. (2002)- Sensitivity- 86.4% & Specificity- 86.1%, Crucitti *et al*. (2003)- Sensitivity- 83.1% & Specificity- 98.5%.

In this study although the wet preparation was performed at the collection site, only 22% of patients with confirmed *T. vaginalis* infection were diagnosed by wet preparation and the sensitivity was lower compared to PCR. Culture as a gold standard for diagnosis of *T. vaginalis* can detect it at 48–72 hours, but it may take up to7 days to obtain the final results (Pillay *et al*. 2004) a delay in therapy while waiting for result is non desirable.

Culture should be used when the wet mount is negative. Here we used culture as a gold standard to determine the sensitivity and specificity of wet preparation and PCR. The reference study to calculate the sensitivity and specificity of culture method in some papers is the culture medium itself, which may yield higher estimate of sensitivity for the culture. (Patel *et al*., 2000).

**Conclusion**

We analysed a number of clinical samples by culture, direct microscopy and PCR, none of the diagnostic assays could detect all positive samples, but PCR showed a higher detection rate than others in detection of *T. vaginalis* in vaginal swab samples. Overall, the sensitivity of the PCR assay resulting from this study was lower than those previously described. These findings could be the result of the nature of the specimen population and suggests a strain variability.

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