

Evaluation of DNA Based Techniques for the Diagnosis of Human Vaginal Trichomoniasis in North Indian Population

Running title: Diagnostic methods for human vaginal trichomoniasis.

ABSTRACT:

Introduction: Human trichomoniasis due to *Trichomonas vaginalis* is a curable sexually transmitted infection. It may lead to symptomatic vaginitis or asymptomatic carrier state. The symptoms and signs mimic other pathologies and conventional techniques of diagnosis have its own limitations. Therefore, the present study aimed to evaluate a newly established in-house PCR based on *pfoB* gene and compared it with conventional wet smear examination method and *18S rRNA* gene based PCR technique for the diagnosis of *Trichomonas vaginalis* in symptomatic and asymptomatic subjects.

Material and Methods: Four hundred women in age group 20-57 years age-range-attending the Obstetrics and Gynecology out Patients Department (OPD) of Nehru Hospital attached to Post Graduate Institute of Medical education and Research (PGIMER) Chandigarh, India were included in the study. Based on the symptoms and signs, 344 (86%) women were categorized as symptomatic and 56 (14%) as asymptomatic. Vaginal swabs collected from all the women were processed by three techniques that is wet smear, *18S rRNA* and the *pfoB* gene based PCR techniques for the detection of *T. vaginalis*.

Results: The main presenting symptom in majority of symptomatic patients was vaginal discharge. The highest numbers of *T. vaginalis* positive patients were found in the sexually active age group of 20 to 40 years. The DNA based diagnostic methods, the *pfoB* and the *18S rRNA* PCR have outperformed and were found to be positive in significantly higher number

27 of subjects (20.7% and 18.6%, respectively) than the wet smear (6.6%) method. The
28 diagnostic efficacy and kappa value by the use of all the three techniques i.e wet mount, 18S
29 rRNA and pfoB (criteria 'a') was found to be between 86.8 and 100%, & and 35.5 and 66%
30 respectively.

31 **Conclusions:** Our data suggests that out of the three techniques used that is wet smear, 18S
32 rRNA and the pfoB gene based PCR techniques the application of any two techniques, out of
33 wet mount, 18S rRNA and pfoB gene based PCR as compared to only wet smear method may
34 serve be useful for the diagnosis of *T. vaginalis* infection in symptomatic and asymptomatic
35 subjects. This information may critically help clinicians to make a timely and accurate
36 diagnosis.

37
38 **KEYWORDS:** *T. vaginalis*, Trichomoniasis, diagnosis, pfoB PCR, 18S rRNA PCR, wet
39 smear examination.
40

41 1. INTRODUCTION

42 Human vaginal trichomoniasis is the most common non-viral sexually transmitted
43 infection (STI) (1) with adverse but significant effect of infection on public health. The
44 prevalence of *Trichomonas* infection in developing countries is increasing every year to the
45 level of approximately 12-30% and is prevalent mainly in women of the sexually active age
46 (1, 2). There is a greater tendency of the *Trichomonas* infected asymptomatic women to
47 become symptomatic (3). A varied clinical presentation of trichomonas involves severe
48 inflammatory manifestation such as urethritis, vulvo-vaginitis, and cervicitis leading to
49 vaginal discharge, itching, dysuria, foul smell and dyspareunia in females. Women with
50 known chronic infection are at greater risks of atypical pelvic inflammatory diseases, HIV
51 infection and cervical cancer (4, 5). An early detection of *T. vaginalis* would be a crucial
52 factor for treatment, reduction and prevention of negative health outcomes in women (6, 7).

A primitive conventional technique for visualization of trichomonas trophozoites is by wet smear examination. This method is quick and cheap but has a limited sensitivity (60-70%). Although, culture technique yields **relatively** higher sensitivity (98.5%) and specificity and is considered as a 'gold standard' for the diagnosis of Trichomoniasis (8). However, it requires well established facilities and expertise, which may not be available in all the diagnostic centres. ~~Moreover, it is suggested that culture should be examined daily for at least 7 days, before labelling it as negative, thus it is time consuming.~~

Various sero-diagnostic techniques have been applied to demonstrate the presence of specific antibodies in serum and vaginal secretions (VS) with variable sensitivity (10) ~~like -~~ ~~Previously, we showed that~~ detection of antibody response to cysteine proteinase 30 (CP30) ~~was highly sensitive and specific assay for the detection of the anti-CP antibody in serum samples from symptomatic and asymptomatic patients~~ (11). *Trichomonas* infection was reported in 36.5% ~~94.2%~~ and 96.7% of women **investigated** by wet smear, ~~culture~~ and ELISA **respectively** ~~of vaginal washes respectively with a specificity of 94.4%~~ (12). ~~Anti-trichomonad IgA antibodies was estimated in serum and VS of symptomatic and asymptomatic T. vaginalis patients before and after treatment.~~ Significantly higher levels of anti-trichomonad IgA antibodies were found in *T. vaginalis* patients as compared to controls (13). Monoclonal antibodies against *T. vaginalis* proteins were also used for detection of Trichomoniasis (14). Though less specific, the rapid XenoStrip-Tv test in vaginal swab samples from women attending **sexually transmitted disease-STI** clinics was found to be more sensitive than the wet smear examination (15). The XenoStrip-Tv test was used, ~~for research studies,~~ but **is could not prove to be** applicable for point-of-care diagnostic assay. Another relatively simple test, the **OSOM (One Step One Minute)** trichomonas rapid test is an immune-chromatographic capillary flow (dipstick) assay used **in-for vaginal swab-VS specimen and showed** a better sensitivity and specificity ~~was reported~~ as compared to the wet

smear examination (16). The only limitations of OSOM test was that it was performed on frozen samples in batches in a research setting. **Therefore** antibody detection assays demands well-established facilities and expertise, which may not be available in all the diagnostic centers.

In order to improve the sensitivity and specificity of the diagnostic techniques, recombinant DNA techniques with high sensitivity have increasingly been used for the diagnosis of microbial infections. Correspondingly, the development of PCR techniques for trichomonas detection have been described, including the use of oligonucleotide probe test with a sensitivity and specificity of 80-90% and 95% respectively (17-19). Another study reported that the affirm-VPIII test in **VS specimens from of** both symptomatic and asymptomatic patients was better **in-for** the detection of vaginitis as compared to wet smear (20). The Affirm assay was more likely to identify Candida and Gardnerella than ~~Trichomonas, and also can have false negative results for Trichomonas in asymptomatic women.~~ Affirm-VPIII is more expensive, and is also classified as a moderately complex test, which is based on the principles of nucleic acid hybridization ~~and uses two distinct single-stranded probes for each organism, a capture probe and a color development probe~~ (20). The newly established pyruvate:ferredoxin oxidoreductase proprotein (*pfoB*) gene has also been exploited for performing *T. vaginalis* specific diagnostic assays (21, **35**). In our earlier report, *pfoB* gene PCR did not show any cross reactivity with the genome of other microorganisms or human DNA (21, **35**). Despite all these efforts, PCR based diagnostic methods are still in experimental stages in the developing countries. Moreover, lack of facilities and relevant expertise in the conventional diagnostic laboratories has impeded the routine use of DNA based techniques.

The *pfoB* gene contains highly conserved DNA sequences that are useful for performing diagnosis assays **(22-24)**. Given the variable sensitivity and specificity of the

applied techniques for the diagnosis, including the DNA based methods (17), the present study aimed to evaluate *pfoB* gene based PCR method in comparison to the earlier applied techniques (18S rRNA PCR and wet smear examination) that were used for the diagnosis of *T. vaginalis*. Further, analysis of various combinations used of these techniques based on the results of individual technique was attempted-carried out to reveal the utility of their application of more than one technique as compared to use of single technique for the diagnosis of symptomatic and asymptomatic *Trichomonas* infection.

2. PATIENTS AND METHODS

2.1 Patients

For the-this study, 400 women between 20-57 years of age, visiting the Obstetrics and Gynecology out Patients Department (OPD), Nehru Hospital attached to the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh (India) were enrolled-in the study. The patients with complaint of vaginal discharge, itching, foul smell, pruritus, dysuria, dyspareunia and abdominal pain were categorized as suggestive of trichomoniasis and labelled as symptomatic. Subjects attending the OPD for routine ante/postnatal follow-up, family planning advice and infertility without any symptoms and signs suggestive of trichomoniasis were labelled as asymptomatic. The per-vaginal (P/V) and per-speculum (P/S) examination of all the women was conducted after obtaining an informed consent and the clinical findings and relevant history was recorded on a pre-planned proforma.

2.2 Sample collection and processing

During P/S-per-speculum examination, two vaginal swabs were collected from posterior vaginal fornix from each subject, using commercially available sterile wet cotton swabs (HiMedia, India). All the vaginal swabs were transported to the department of Medical Parasitology, PGIMER within half an hour of collection. The first swab was subjected to wet

smear examination and the second swab was stored at -20°C for DNA extraction ~~and *T. vaginalis* detection by PCR. The second swab was transported under refrigerated conditions to ACBR, Delhi for extraction of DNA~~ for PCR methods.

2.3 Wet smear examination

The wet smear examination was performed to detect the presence of motile Trichomonads. For this, a drop of normal saline (0.90% NaCl) was put on a glass slide and the swab was gently rolled in the saline to make a thin smear. A clear coverslip was placed over the smear and examined under a light microscope for the presence of *T. vaginalis* as described previously (8).

2.4 Genomic DNA isolation and Polymerase Chain Reaction

~~A frozen wet swab was thawed and squeezed into 1ml of chilled 1x PBS. The sample was mixed thoroughly by vortexing. The sample was centrifuged at 14,000xg at 4°C and the pellet was suspended in 40µl of 1x chilled PBS.~~ The total genomic DNA was isolated from all the samples as described earlier (21, 25, 35). In brief, phosphate buffered saline (PBS, 1mL) was added to the vial containing dry swabs and incubated for 10 minute at 4°C, mixed by vortexing thoroughly and squeezed. An aliquote of the sample (400µl) was centrifuged at 11,000 X g at 4°C for 10 minutes. The supernatant was discarded and the cell pellet was suspended in 40µl of PBS followed by centrifugation at 11,000 X g at 4°C for 5 minutes. Total genomic DNA was isolated essentially as described earlier from the laboratory for other pathogens (21, 25, 35). Nucleic acids were either stored at 4°C ~~til~~ for further use or used directly as template DNA (5µl) for PCR assay. Thereafter *T. vaginalis* detection was performed by PCR using oligonucleotides specific to *18S rRNA* (16S-like ribosomal gene; GenBank accession number U17510) and *pfoB* gene (about 3.7kb length; GenBank accession number U16823) of *T.*

vaginalis. The genomic DNA isolated from vaginal swabs was used as a template. An amplicon of 312bp and 338bp sizes were obtained for *18S rRNA* (17) and *pfoB* gene respectively. For *pfoB* gene, the amplification was carried out in a 25µl volume reaction mixture containing 1xTaq DNA Polymerase Buffer, 200µM of the deoxyribonucleoside triphosphates (Bangalore Genei, India), 5 pmoles of forward (pfoBF 5'-CAAAGTCAACATGGCTATGAT-3') and reverse primer (pfoBR 5'-GAAGACCTGTGTGGATGGATGT-3'), 5µl of genomic DNA sample, 1.0U of Taq DNA Polymerase (Bangalore Genei, India). PCR included a negative control (sterile water) and a positive control (1ng of purified genomic DNA of *T. vaginalis*). After an initial denaturation of 96°C for 5 minute, PCR amplification was achieved in a thermal cyclers (I-cycler, BioRad, USA) for 35 cycles using following conditions; cycling at 96°C for 20 seconds, 56°C for 30 seconds and 72°C for 25 seconds. A final extension was performed at 72°C for 7 minutes. The amplicons (20µl) were analyzed by electrophoresis in a TAE buffer using 2% agarose gel containing 0.5µg/ml of ethidium bromide. After electrophoresis, the DNA bands were visualized on a UV transilluminator (Alpgen, USA). Figure_1 shows amplification of *T. vaginalis* in clinical samples using primers specific to *18S rRNA* and *pfoB* gene.

2.5 Statistics and calculations

~~A test of proportion was used to assess the patient positive for *T. vaginalis* in the symptomatic and the asymptomatic group.~~ A Chi-square test of homogeneity and Kruskal-Wallis test were used to analyze for the statistical analysis distribution of symptomatic patients in reference to their symptoms. The Kruskal Wallis test was used to analyze the statistical difference in the number of patient complaints between different age groups. ~~A test of proportion was used to assess the proportion of the subjects positive for *T. vaginalis* using various diagnostic tests.~~ PCR data was analyzed using InStat programme. Standard formulae

were used to make a comparison of the sensitivity, specificity and positive/negative predictive values of the diagnostic methods (26, 27). Due to lack of an efficient Gold Standard Criteria for diagnosis of *T. vaginalis* and lower sensitivity of wet smear examination, we evaluated the diagnostic efficacy and kappa value of *T. vaginalis* by multiple techniques in various combinations. The diagnostic efficacy and kappa value of the techniques was calculated after defining the best possible measure of 'true positives' as criteria, 'a'; Positive by all the three techniques i.e wet mount, *18S rRNA* and *pfoB*.

For this, following four discrete measures were defined.

- a). Positive by all the three techniques i.e; wet mount, *18S rRNA* and *pfoB*
- b). Positive by two techniques (wet mount and *18S rRNA PCR*)
- c). Positive by two techniques (wet mount and *pfoB PCR*)
- d). Positive by two PCR techniques (*18S rRNA* and *pfoB*)

2.6 Ethical Approval

The institutional ethical committee of Dr. B.R Ambedkar Centre for Biomedical Research, University of Delhi, Delhi, India (EC. No: F-50-2/Eth.Com/ACBR/11/2110) and Postgraduate Institute of Medical Education and Research, Chandigarh, India (EC. No: PGI/IEC/2012/1549-50) approved this project. **Written consent** was obtained from all the adult subjects included in this study.

3. RESULTS

3.1 Subjects and Symptomatology

A total of 344 patients presented with symptoms (vaginal discharge, itching, **pruritus**, foul smell, dyspareunia, and/or abdominal pain) suggestive of trichomoniasis were labelled as

symptomatic subjects and 56 subjects without any symptoms and signs suggestive of trichomoniasis were labelled as asymptomatic. The main presenting symptom noted was vaginal discharge in 305 (76.25%), followed by vaginal discharge and itching in 159 (39.75%), vaginal discharge, itching and pruritus in 70 (17.5%), vaginal discharge, itching, pruritus, and dyspareunia in 44 (11%), vaginal discharge, itching, pruritus, dyspareunia and abdominal pain in 36 (9%) patients (Table 1). All the asymptomatic subjects (56) had a normal appearing vagina and cervix. Assessment of the symptoms in the symptomatic group of patients was made using Chi-square test of homogeneity. The distribution of symptomatic patients with reference to their symptoms was found to be homogenous (Chi-square=621.50) and significant ($p=0.000001$).

3.2 Clinical findings of the symptomatic subjects

Patients were categorized into four age groups (20-30, 31-40, 41-50 and >50 years) in order to find out which sexually active age group is most vulnerable to *T. vaginalis* infection. Clinical details of the symptomatic patients are summarized in Table 2. ~~The Kruskal Wallis test was used to compare the difference between patient's complaints and signs based on physical examination, irrespective of which type of complaints have been received. There was a significant difference in complaints and signs of the various age groups. However, the distribution of the complaints and signs of the patients based on physical examination was found to be similar within each age group. The analysis of complaints among all the age groups was found to be significant ($p=0.0007$).~~ Subjects belonging to the age group of 20-30 (n=122) and 31-40 years (n=119) showed more symptoms and signs as compared to the age group 41-50 (n=82) and >50 (n=21) suggestive of trichomoniasis. Vaginal discharge was the main presenting complaint followed by abdominal pain, itching and foul smell among all the age groups. ~~P/V Per vaginal~~ examination ~~(P/V)~~ of symptomatic patients showed

inflammation/erosion in 16 (2.6%) followed by uterus bulky 10 (3.2%). Similarly, a ~~P/Sper-~~
~~speculum~~ examination of symptomatic patients suggested cervix (Cx) erosion as a clear
clinical indication in 114 (33.7%), followed by Cx bleeding in 31 (9%), strawberry Cx in 10
(2.9%), multiparous Cx in 2 (0.6%) and normal Cx in 187 (53.8%) (Table-2).

3.3 Distribution of symptoms of the symptomatic patients in various age groups

Significantly, ~~more-higher~~ number of women in the ~~sexually active age~~ groups of 20-30 and
31-40 years presented ~~with~~ the clinical complaints ~~suggestive of trichomoniasis~~ (Table 2).
Therefore, for further statistical analysis these age groups were combined and labeled as 20-
40 years age group. Among the symptomatic patients, the frequency of presenting symptoms
in age group 20-40 years were significantly higher than in the age groups 41-50 and >50
years ($p<0.01$; ~~Figure 2A~~). ~~Per Vaginal examination of the uterus (Ut) of symptomatic~~
~~patients showed no significant signs in the age group 20-40 as compared to the other two age~~
~~groups. P/S~~ ~~Per Speculum~~ examination of the ~~cervix (Cx)~~ of symptomatic patients showed
that Cx erosion was the main signs in the age group 20-40 years while the frequency of Cx
bleeding and strawberry were significantly higher in the age groups >50 years ($p<0.01$;
~~Figure 2B~~). ~~P/V examination of the uterus (Ut) of symptomatic patients showed no~~
~~significant signs in the age group 20-40 as compared to the other two age groups (Figure 2C).~~

3.4 Detection of *T. vaginalis* by wet smear examination, *18S rRNA* and *pfoB* PCR

Samples collected from all the subjects were examined for *T. vaginalis* detection by wet
smear, *18S rRNA* and *pfoB* gene based PCR methods. ~~Test of proportion was used following~~
~~a null hypothesis for checking the efficiency of individual diagnostic technique.~~ The
percentage of positivity in the asymptomatic group was higher than the symptomatic by *pfoB*
method ($p=0.037$) and was non-significant ($p=0.08$) by *18S rRNA* PCR method (Figure ~~32~~A).

Out of 400 women, wet smear, *18S rRNA* PCR and *pfoB* PCR was positive in 12 (3%), 28 (7%) and 30 (7.5%) women respectively.

By wet smear examination, 10 (2.91%) out of 344 symptomatic and 2 (3.57%) out of 56 asymptomatic women were found positive for *T. vaginalis* suggesting a similar proportion of positive patients in both the groups ($p=0.27$;NS). However, *18S rRNA* PCR method showed a higher efficiency, by which 21 (6.1%) symptomatic and 7 (12.5%) asymptomatic women were positive, suggesting the proportion of positive patients in the later group was higher but not significant ($p=0.08$;NS). Interestingly, the *pfoB* PCR showed 22 (6.4%) symptomatic and 8 (14.3%) asymptomatic women positive, suggesting the proportion of positive patients in the latter group was significantly higher ($p=0.03$) (Figure 32 A).

In order to decipher the status of *T. vaginalis* infection among different age groups, the frequency of *T. vaginalis* detection by the various methods was calculated. It was found that the highest numbers of *T. vaginalis* positive patients were in the sexually active age group 20-40 (Figure 32B).

3.5 Sensitivity, specificity, positive and negative predictive value and diagnostic efficacy of the techniques

Based on the best possible measure of 'true positive' as criteria 'a'; 'Positive by all the three techniques (wet mount, *18S rRNA*, *pfoB*)', the sensitivity of the wet smear ('a'), *18S rRNA* ('a') and *pfoB* ('a') was 100% each in samples from symptomatic and asymptomatic subjects respectively. Specificity of the wet smear was 98.4% and 100% in symptomatic and asymptomatic subjects respectively. Specificity of *18S rRNA* and *pfoB* based DNA detection methods was 95.1% and 94.8 in symptomatic and 90.2% and 88.5% in asymptomatic subjects respectively (Table 3). The data suggests that the kappa value and diagnostic efficacy of

criteria 'a'; ~~Positive by all the three techniques (wet smear, 18S rRNA, pfoB)~~ can be considered as the best combination for the diagnosis.

4. DISCUSSION

In the present study, the main presenting symptoms among the symptomatic group were found to be vaginal discharge, followed by itching, pruritus, foul smell, dyspareunia and abdominal pain. The speculum examination showed cervicitis as the main presenting sign followed by vaginitis in the symptomatic patients, while all the asymptomatic subjects showed normal cervix and vagina. The status of presenting symptoms based on the age groups of *T. vaginalis* patients has not been delineated well before. This aspect of population study throws light on 'which age group of patients is more prone to *trichomonas* infection' in order to target a specific population age group for diagnosis. Significantly higher number of women in the sexually active age group of 20-40 years presented with the clinical complaints suggestive of trichomoniasis, compared to other age groups. This is understandable since this age group represents the active reproductive phase amongst women (1).

A rapid and efficacious diagnostic test can be critical for the control of STIs like trichomoniasis. Wet smear and culture methods are the most frequently used methods of diagnosis for *T. vaginalis* detection. However, time limit for wet smear examination, following sample collection and lower sensitivity limits its use as an effective diagnostic technique. ~~The culture technique, although highly sensitive, has its own limitations including the requirement of infrastructure facilities for culture and expertise besides being time consuming process.~~ Therefore, developing countries have adopted a diagnosis system based on symptoms and signs of women as the primary strategy for management of trichomoniasis. However, up to 50% of *T. vaginalis* infections may be asymptomatic leading to considerable

under-treatment (28).

In the study conducted in India, the prevalence of *T. vaginalis* infection in Mysore (South India) was 8.5% (29), in Chandigarh (North India) was 3.6% in symptomatic and 2.7% in asymptomatic cases positive by wet smear examination (30). In the present study when both the groups were taken together percentage positivity by *pfoB* and the *18S rRNA* PCR were significantly higher 20.7% and 18.6%, respectively as compared to wet smear examination. Though the PCR for detection of *T. vaginalis* in vaginal washes VS showed moderately high sensitivity (80-95%) as compared to wet smear examination (31-32). The only diagnostic assay for detection of *T. vaginalis* cleared by the FDA is NAAT aptima *T. vaginalis* assay (33). The Aptima (TMA) *T. vaginalis* assay is a nucleic acid amplification test that utilizes target capture, transcription-mediated amplification, chemiluminescent probe hybridization to detect *T.vaginalis 18s rRNA* and a high sensitivity 96.7% and specificity 97.5% has been reported (34). ~~This study was conducted for the detection of *T. vaginalis* in self-collected first catch urine specimens and clinician-collected endo-cervical swab, vaginal swab, and endo-cervical thin prep samples (33). The OSOM, a rapid antigen test is an immune-chromatographic capillary flow enzyme immunoassay dipstick test performed on vaginal secretions. It takes 10 minutes and works fine with live or dead organisms. The~~ sensitivities of the OSOM rapid antigen test varied from 82-92.5% and specificity was 100% (34). ~~Antigen detection test is rapid but is cost effective. It is difficult to detect when infection rate is low or in asymptomatic cases or if swab is not properly obtained (34).~~ Although DNA based diagnostic tests have improved the sensitivity of trichomonas diagnosis (19), yet these are still in limited use in the developing countries. Therefore, the evaluation of newer DNA based diagnostic tests for screening of human trichomoniasis in symptomatic and asymptomatic subjects is of significance from public health importance.

Significantly higher sensitivity and specificity of DNA based diagnostic methods including the PCR have been reported earlier. It is suggested that the PCR based diagnostic methods are important tools in aiding epidemiologic evaluations based on prevalence and morbidity of the disease (32). Therefore the main aim of this study was to evaluate newly established *pfoB* gene based PCR methods in combination with the known conventional techniques (wet smear) in symptomatic and asymptomatic subjects. *pfoA* is a *T. vaginalis* specific hypothetical gene which translate into a cell-binding protein and is involved in adherence of *T. vaginalis* to host's vaginal epithelial cells (23, 24). The *pfoB* has a highly conserved and unique DNA sequences that was exploited for performing *T. vaginalis* specific diagnostic assays. Previously we found that *pfoB* gene is specific for *T. vaginalis* and does not show any cross reactivity with the genome of other microorganisms like *T. gallinae*, *T. tenax*, *Tritrichomonas foetus*, *Chlamydia pneumoniae*, *C. trachomatis*, *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Herpes simplex virus 1&2*, *Human papilloma virus L2*, *Mycobacterium tuberculosis*, *Candida albicans* and Human DNA samples (21, 35).

In the present study, an efficiency of the techniques was calculated and only 3.2% were found positive by wet smear method, thus, in both symptomatic and asymptomatic groups, large numbers of patients detected positive by PCR were found negative by wet smear. Intriguingly, we also observed that a percentage of *T. vaginalis* infection detected by DNA based diagnostic methods among the symptomatic patients was less (6.4%) as compared to asymptomatic women (14.3%). One probable reason for a less percentage of positivity among the symptomatic group could be prior treatment of symptomatic patients with metronidazole, as described earlier (36). Overall, the *pfoB* gene followed by *18S rRNA* gene PCR showed the highest positivity among the *T. vaginalis* infected patients as compared

to the wet smear examination method. Positive by any two techniques out of wet mount, *18S rRNA* and *pfoB* detection by PCR was found to be from 46% to 100% in both symptomatic and asymptomatic subjects. Thereby ~~the~~our study suggests that the diagnostic efficacy and kappa value of criteria ‘a’ can be termed as the best combination as a diagnostic tool. This knowledge may further add to the prevalence and the comparative diagnosis as a predictor of the *Trichomonas* infection, which could be helpful in the management of Trichomoniasis.

5. CONCLUSION

Our findings suggest that the vaginal discharge is the main presenting symptom in majority of symptomatic patients. The highest number of *T. vaginalis* positive patients was found to be in the **sexually active age** group of 20 to 40 years. The *pfoB* and *18S rRNA* gene based PCR techniques are more efficient as compared to traditional wet smear examination. *T. vaginalis* infected asymptomatic subjects are known to spread infection to their sexually active partners, therefore the proposed set of methods for diagnosis may prove to be a robust method for identification and early detection of *T. vaginalis* positivity among the asymptomatic subjects as well. These results showed that the PCR techniques (both *18S rRNA* and *pfoB*) are better for detection of *T. vaginalis* as compared to the conventional wet smear examination. **It would be worthwhile to encourage further studies to explore the feasibility and cost-effectiveness of these diagnostic tests.**

REFERENCES

- 374 1. Fichorova RN. Impact of *T. vaginalis* infection on innate immune responses and
375 reproductive outcome. Journal of reproductive immunology. 2009;83(1-2):185-9.
- 376 2. Sutton M, Sternberg M, Koumans EH, McQuillan G, Berman S, Markowitz L. The
377 Prevalence of *Trichomonas vaginalis* Infection among Reproductive-Age Women in
378 the United States, 2001–2004 Clinical Infectious Diseases. 2007; 45:1319–26.
- 379 3. Wendel KA. Trichomoniasis: What's New? Current infectious disease reports.
380 2003;5(2):129-34.
- 381 4. Lazenby GB, Taylor PT, Badman BS, Mchaki E, Korte JE, Soper DE, Pierce JY. An
382 Association Between *Trichomonas vaginalis* and High-Risk Human Papilloma virus in
383 Rural Tanzanian Women Undergoing Cervical Cancer Screening. Clinical
384 Therapeutics. 2014; 36(1): 38-45.
- 385 5. Shafir SC, Sorvillo FJ, Smith L. Current issues and considerations regarding
386 trichomoniasis and human immunodeficiency virus in African-Americans. Clinical
387 microbiology reviews. 2009;22(1):37-45.
- 388 6. Yadav M, Verma N, Dhanda RS. Impact of sexually transmitted infections on women
389 health. Health. 2013;5(8):11.
- 390 7. Malla N, Goyal K, Dhanda RS, Yadav M. Immunity in urogenital protozoa. Parasite
391 immunology. 2014;36(9):400-8.
- 392 8. Sharma P, Malla N, Gupta I, Ganguly NK, Mahajan RC. A comparison of wet mount,
393 culture and enzyme linked immunosorbent assay for the diagnosis of trichomoniasis in
394 women. Tropical and geographical medicine. 1991;43(3):257-60.
- 395 9. Kissinger PJ, Dumestre J, Clark RA, Wenthold L, Mohammed H, Hagensee ME, et al.
396 Vaginal swabs versus lavage for detection of *Trichomonas vaginalis* and bacterial

- 397 *vaginosis* among HIV-positive women. Sexually transmitted diseases. 2005;32(4):227-
398 30.
- 399 10. Mason PR, Gregson S, Gwanzura L, Cappuccinelli P, Rapelli P, Fiori PL. Enzyme
400 immunoassay for urogenital trichomoniasis as a marker of unsafe sexual behaviour.
401 Epidemiology and infection. 2001;126(1):103-9.
- 402 11. Yadav M, Dubey ML, Gupta I, Malla N. Cysteine proteinase 30 (CP30) and antibody
403 response to CP30 in serum and vaginal washes of symptomatic and asymptomatic
404 *Trichomonas vaginalis*-infected women. Parasite immunology. 2007;29(7):359-65.
- 405 12. Tawfeek GM, Oteifa NM, el-Gozamy BR. Evaluation of an IgG cystatin capture
406 enzyme-linked immunosorbent assay for the detection of anti-cysteine proteinase
407 antibodies in asymptomatic trichomoniasis patients. Journal of the Egyptian Society of
408 Parasitology. 2003;33(1):67-83.
- 409 13. Sharma P, Malla N, Gupta I, Ganguly NK, Mahajan RC. Antitrichomonal IgA antibodies
410 in trichomoniasis before and after treatment. Folia Microbiol 1991; 36: 302–304.
- 411 14. Yadav M, Dubey ML, Gupta I, Bhatti G, Malla N. Cysteine proteinase 30 in clinical
412 isolates of *T. vaginalis* from symptomatic and asymptomatic infected women.
413 Experimental parasitology. 2007;116(4):399-406.
- 414 15. Kurth A, Whittington WL, Golden MR, Thomas KK, Holmes KK, Schwebke JR.
415 Performance of a new, rapid assay for detection of *Trichomonas vaginalis*. Journal of
416 clinical microbiology. 2004;42(7):2940-3.
- 417 16. Huppert JS, Batteiger BE, Braslins P, Feldman JA, Hobbs MM, Sankey HZ, et al. Use
418 of an immunochromatographic assay for rapid detection of *Trichomonas vaginalis* in
419 vaginal specimens. Journal of Clinical Microbiology. 2005;43(2):684-7.

- 420 17. Mayta H, Gilman RH, Calderon MM, Gottlieb A, Soto G, Tuero I, et al. 18S ribosomal
421 DNA-based PCR for diagnosis of *Trichomonas vaginalis*. Journal of clinical
422 microbiology. 2000;38(7):2683-7.
- 423 18. Lawing LF, Hedges SR, Schwebke JR. Detection of trichomonosis in vaginal and urine
424 specimens from women by culture and PCR. Journal of clinical microbiology.
425 2000;38(10):3585-8.
- 426 19. Dwivedi SP, Husain N, Singh RB, Malla N. 18S ribosomal DNA based PCR diagnostic
427 assay for *Trichomonas vaginalis* infection in symptomatic and asymptomatic women in
428 India. Asian Pacific Journal of Tropical Disease. 2012;2012:133-8.
- 429 20. Brown HL, Fuller DD, Jasper LT, Davis TE, Wright JD. Clinical evaluation of affirm
430 VPIII in the detection and identification of *Trichomonas vaginalis*, *Gardnerella*
431 *vaginalis*, and *Candida* species in vaginitis/vaginosis. Infectious diseases in obstetrics
432 and gynecology. 2004;12(1):17-21.
- 433 21. Saluja D, Sonkar SC, Mishra PK. A Molecular Beacon-mediated Oligonucleotide-
434 based Diagnostic Probe/Tool/Kit for Detection of *Trichomonas vaginalis* and Method
435 of Working for same. Indian Patent. 2014;No. 1098/DEL/2013.
- 436 22. Hrdy I, Muller M. Primary structure and eubacterial relationships of the
437 pyruvate:ferredoxin oxidoreductase of the amitochondriate eukaryote *Trichomonas*
438 *vaginalis*. Journal of molecular evolution. 1995;41(3):388-96.
- 439 23. Brito VM, Gómez CY, Cervantez PM, González LA, Rodríguez MA, López JO, Robles
440 AG, Arroyo R. A *Trichomonas vaginalis* 120 kDa protein with identity to
441 hydrogenosome pyruvate:ferredoxin oxidoreductase is a surface adhesin induced by
442 iron. Cell Microbiology. 2005;7(2):245-58.

- 443 24. Cervantez PM, Robles AG, Guerra REC, López JO, Saavedra E, Pineda E, Arroyo R.
444 Pyruvate:ferredoxin oxidoreductase (PFO) is a surface-associated cell-binding protein
445 in *Trichomonas vaginalis* and is involved in trichomonal adherence to host
446 cells. Microbiology. 2011;157(Pt 12):3469-82.
- 447 25. Patel AL, Sachdev D, Nagpal P, Chaudhry U, Sonkar SC, Mendiratta SL, et al.
448 Prevalence of Chlamydia infection among women visiting a gynaecology outpatient
449 department: evaluation of an in-house PCR assay for detection of *Chlamydia*
450 *trachomatis*. Annals of clinical microbiology and antimicrobials. 2010;9:24.
- 451 26. Kaushik K, Khurana S, Wanchu A, Malla N. Evaluation of staining techniques, antigen
452 detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive
453 and seronegative patients. Acta tropica. 2008;107(1):1-7.
- 454 27. Krieg AF, Gambino R, Galen RS. Why are clinical laboratory tests performed? When
455 are they valid? JAMA: the journal of the American Medical Association.
456 1975;233(1):76-8.
- 457 28. Sobel JD. What's new in bacterial vaginosis and trichomoniasis? Infectious disease
458 clinics of North America. 2005;19(2):387-406.
- 459 29. Madhivanan P, Bartman MT, Pasutti L, Krupp K, Arun A, Reingold AL, Klausner JD.
460 Prevalence of *Trichomonas vaginalis* infection among young reproductive age women
461 in India: implications for treatment and prevention. Sex Health. 2009;6(4):339–344.
- 462 30. Kaur S, Khurana S, Bagga R, Wanchu A, Malla N. Trichomoniasis among women in
463 north india: A hospital based study. Indian J sex Transm Dis and AIDS. 2008;29:76-81.
- 464 31. Nye MB, Schwebke JR, Body BA. Comparison of APTIMA *Trichomonas vaginalis*
465 transcription-mediated amplification to wet mount microscopy, culture, and polymerase

- chain reaction for diagnosis of trichomoniasis in men and women. American journal of obstetrics and gynecology. 2009;200(2):188 e1-7.
32. Wendel KA, Erbeling EJ, Gaydos CA, Rompalo AM. *Trichomonas vaginalis* polymerase chain reaction compared with standard diagnostic and therapeutic protocols for detection and treatment of *vaginal trichomoniasis*. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2002;35(5):576-80.
33. Schwebke JR, Hobbs MM, Taylor SN, Sena AC, Catania MG, Weinbaum BS, et al. Molecular testing for *Trichomonas vaginalis* in women: results from a prospective U.S. clinical trial. Journal of clinical microbiology. 2011;49(12):4106-11.
34. Huppert J S, Mortensen J E, Reed JL, Kahn JA, Rich KD, Miller WC, Hobbs MM. Rapid Antigen Testing Compares Favorably with Transcription-Mediated Amplification Assay for the Detection of *Trichomonas vaginalis* in Young Women. Clinical Infectious Diseases. 2007;45:194–8.
35. Sonkar S C, Sachdev D, Mishra P K, Kumar A, Mittal P, Saluja D. A molecular-beacon-based asymmetric PCR assay for easy visualization of amplicons in the diagnosis of trichomoniasis. Biosensors and Bioelectronics. 2016;86:41–47.
36. Van Der Pol B, Williams JA, Orr DP, Batteiger BE, Fortenberry JD. Prevalence, incidence, natural history, and response to treatment of *Trichomonas vaginalis* infection among adolescent women. The Journal of infectious diseases. 2005;192(12):2039-44.

Table 1: Presenting symptoms in *T. vaginalis* infected symptomatic patients.

S. No	Symptoms	Symptomatic	Asymptomatic
-------	----------	-------------	--------------

		N=344 (86%)	N=56 (14%)
1.	Normal	0	56 (14 %)
2.	Vaginal Discharge	305 (76.25 %)	0
3.	Vaginal Discharge + Itching	159 (39.75 %)	0
4.	Vaginal Discharge + Itching + Pruritus	70 (17.5 %)	0
5.	Vaginal Discharge + Itching + Pruritus + Dyspareunia	44 (11 %)	0
6.	Vaginal Discharge + Itching + Pruritus + Dyspareunia + Abdominal Pain	36 (9 %)	0

Table 2: Clinical details of the symptomatic patients with respect the different age groups.

Age groups (years)	20-30	31-40	41-50	> 50
--------------------	-------	-------	-------	------

		N=344 (%)	N=122 (%)	N=119 (%)	N=82 (%)	N=21 (%)
Clinical profiles						
<i>Complaints</i>						
	Vaginal discharge	305 (88.7)	116 (95.1)	108 (90.7)	71 (86.6)	10 (47.6)
	Itching	141 (41.0)	45 (36.9)	56 (47.1)	33 (40.2)	7 (33.3)
	Pruritus-vulva	95 (27.6)	29 (23.8)	35 (29.4)	28 (34.1)	3 (14.3)
	Dysuria	91 (26.4)	29 (23.8)	33 (27.7)	25 (30.5)	4 (19.0)
	Dyspareunia	62 (18.0)	26 (21.3)	23 (19.3)	11 (13.4)	2 (9.5)
	Abdominal pains	195 (56.7)	76 (62.3)	73 (61.3)	39 (47.6)	7 (33.3)
	Foul smell discharge	131 (38.1)	52 (42.6)	49 (41.1)	24 (29.3)	6 (28.6)
Physical Examination						
<i>P/S</i>	Cx normal	187 (53.8)	66 (54.1)	70 (58.8)	41 (50)	10 (47.6)
	Cx erosion	114 (33.7)	40 (32.8)	35 (29.4)	32 (39)	7 (33.3)
	Cx bleeding	31 (9.0)	7 (5.7)	9 (7.6)	12 (14.6)	3 (14.3)
	Cx strawberry	10 (2.9)	2 (1.6)	1 (0.8)	3 (3.6)	4 (19)
	Cx multiparous	2 (0.6)	0	1 (0.8)	1 (1.2)	0
<i>P/V</i>	Ut normal	306 (91.9)	103 (84.4)	102 (85.7)	80 (97.5)	21 (100)
	Ut mobile	8 (1.7)	3 (2.5)	4 (3.4)	1 (1.2)	0
	Ut bulky	10 (3.2)	2 (1.6)	5 (4.2)	2 (2.4)	1 (4.8)
	Ut enlarged	4 (0.6)	2 (1.6)	1 (0.8)	1 (1.2)	0
	Ut inflamed/erosion	16 (2.6)	6 (4.9)	5 (4.2)	4 (4.9)	1 (4.8)

Abbreviations: P/S, Per-Speculum examination; P/V, Per-Vaginal examination; Cx, cervix; Ut, uterus.

542 **Table 3:** Comparative analysis of wet smear examination, *18S rRNA* and *pfoB* gene based
543 diagnostic methods for *T. vaginalis* detection in symptomatic (N= 53) and asymptomatic (N
544 =17) cases based on four (a to d) criteria of ‘true positives’.

545

S. No	Techniques	Samples	No. of patient (%)	Sensitivity (%)		Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Diagnostic efficacy (%)	Agreement with ‘gold standard’ (kappa value)
1	Wet mount	Symptomatic	10 (2.9%)	a	100	98.4	50	100	98.5	66
				b	100	98.4	50	100	98.5	66
				c	100	99.4	80.6	100	99.4	86.6
				d	35.7	98.4	97.2	50	95.8	39.5
		Asymptomatic	2 (3.57%)	a	100	100	100	100	100	54.6
				b	100	100	100	100	100	100
				c	100	88.5	25	100	88.9	100
				d	40	100	100	93.9	90.1	54.6
2	<i>18S rRNA</i>	Symptomatic	21 (6.10%)	a	100	95.1	23.8	100	95.2	36.9
				b	100	95.1	23.8	100	95.2	36.9
				c	62.5	95.1	23.8	99.0	94.4	32.2
				d	100	97.8	66.7	100	97.9	78.9
		Asymptomatic	7 (12.5%)	a	100	90.2	28.6	100	86.8	41
				b	100	90.2	28.6	100	88.8	41
				c	100	90.2	28.6	100	86.8	41
				d	100	90.2	28.6	100	86.8	81.3
3	<i>pfoB</i>	Symptomatic	22 (6.39%)	a	100	94.8	22.7	100	94.9	35.5
				b	100	94.8	22.7	100	94.9	35.5
				c	100	95.7	36.4	100	95.8	51.6
				d	100	97.5	63.6	100	97.6	76.6
		Asymptomatic	8 (14.28%)	a	100	88.5	25	100	88.9	36.2
				b	100	88.5	25	100	88.5	36.2
				c	40	100	100	93.9	90.2	36.2
				d	100	93.9	62.5	100	94.4	74

546 (a) Positive by all the three techniques i.e wet mount, *18S rRNA*, *pfoB* detection by PCR.

547 (b) Positive by only wet mount and *18S rRNA*

548 (c) Positive by only wet mount and *pfoB*

549 (d) Positive by only *18S rRNA* and *pfoB*

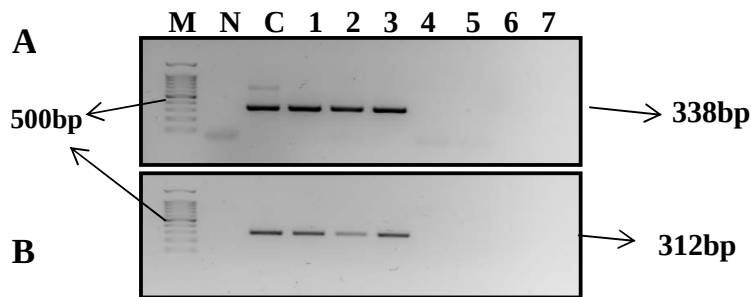


Figure 1. Agarose gel showing amplification of *pfoB* (A) and *18S rRNA* (B) gene of *T. vaginalis* in from randomly selected clinical samples using primers specific to *pfoB* (A) and *18S rRNA* (B) gene. Lane M: 100bp DNA marker, Lane N: Negative control, Lane C: Positive control, Lanes 1-7: Samples from *T. vaginalis* infected patients. In Panel A; Lane 1-3 are TV (+) and Lane 4-7 are TV (-) with *pfoB* while in Panel B; Lane 1-3 are TV (+) and Lane 4-7 are TV (-) with *18S rRNA* gene.

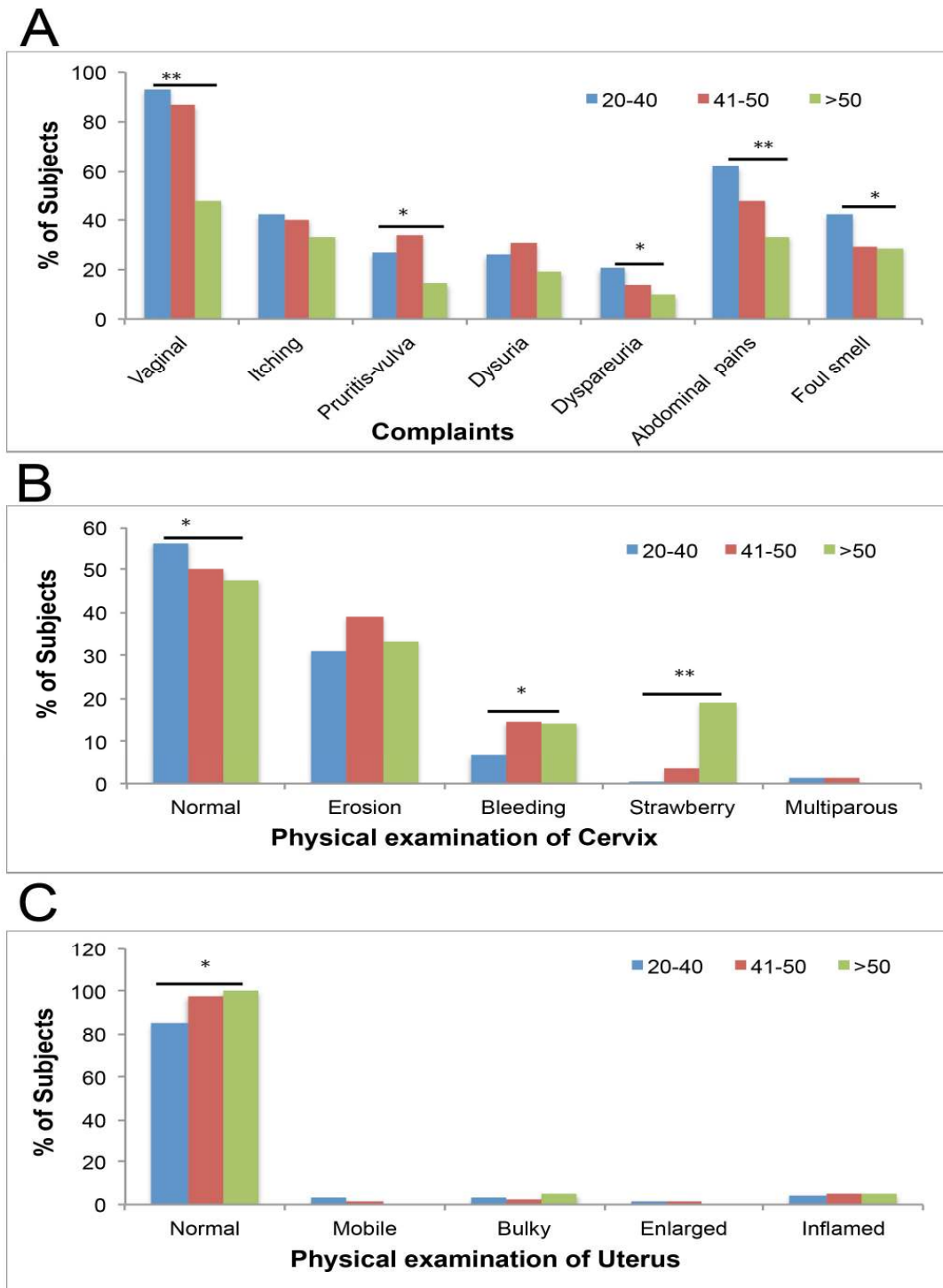


Figure 2. Distribution of symptoms of the symptomatic patients in the age groups 20-40, 41-50 and >51years. Distribution of the percentage of patients was assessed with respect to the type of complaints (A) received, physical examination of the cervix (B), and uterus (C). P values are * p<0.05, **p<0.01.

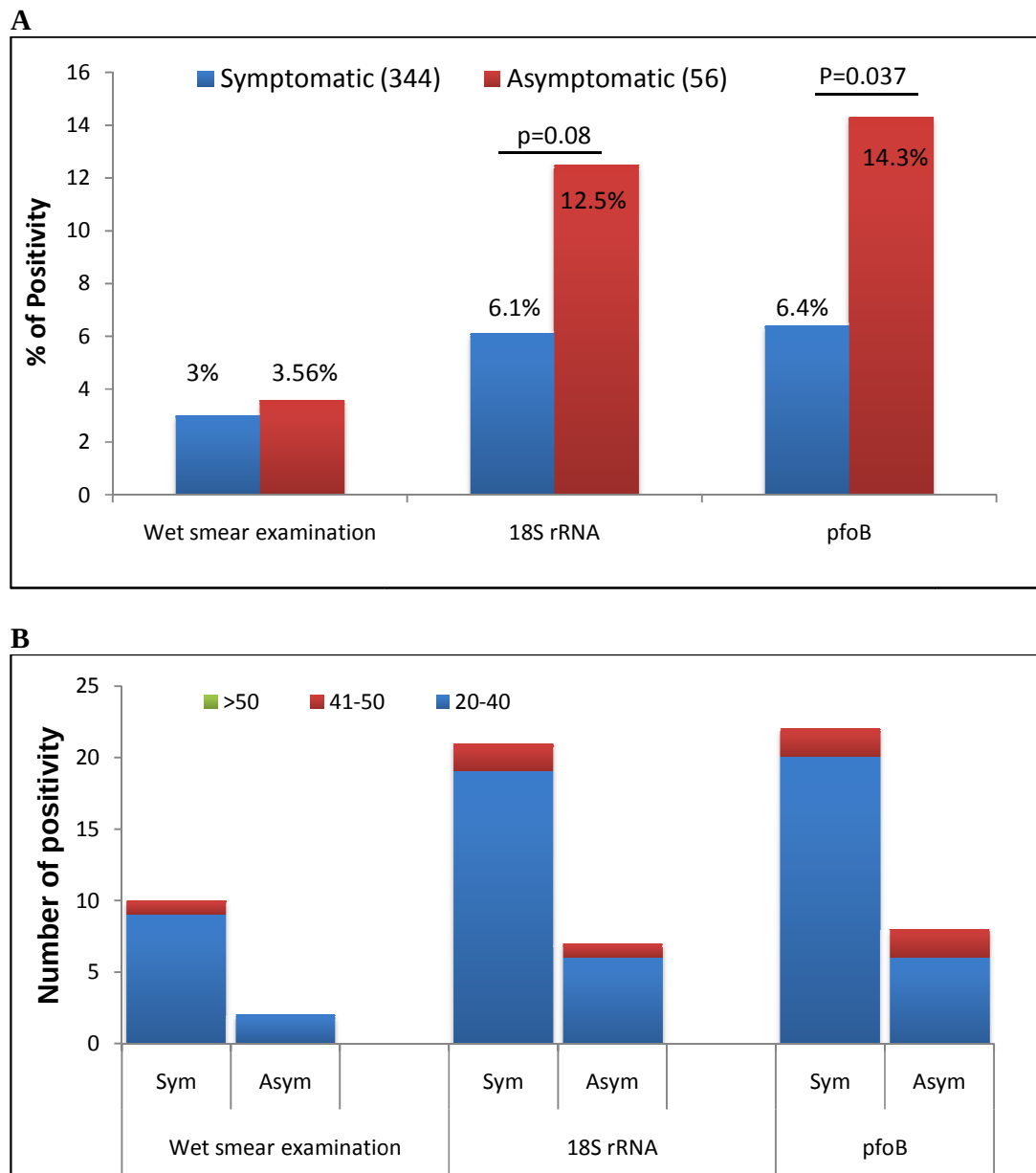


Figure 3. Detection of *T. vaginalis* by various methods (N=400). (A) Percentage of positivity in both Symptomatic and Asymptomatic women. (B) Number of positive patients is graphically represented with respect to age-groups in both Symptomatic and Asymptomatic women.