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Signature of real-time PCR in detection of *Trichomonas vaginalis* infection and its association with human papillomavirus genotype 16

E.A. HEIKAL[1], A.M. ELAMIR[2], M.A. HEGAZI[1], H.S. SALEM[1], A.M. TAWFEIK[3,7], A.H. BOSILAH[4], M.S. BAKRY[5], Y.N. ABDEL-HAFEZ[6]

[1]Department of Medical Parasitology, Faculty of Medicine, Al-Azhar University, Cairo, Egypt
[2] Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[3]Department of Microbiology, Faculty of Medicine, Al-Azhar University, Cairo, Egypt
[4]Department of Obstetrics and Gynecology, Faculty of Medicine, Damietta University, Damietta, Egypt
[5]Department of Obstetrics and Gynecology, [6]Department of Parasitology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[7]Department of Microbiology and Immunology, Faculty of Medicine, Badr University, Cairo, Egypt

Abstract. – OBJECTIVE: **Infection with *Trichomonas vaginalis* (TV) is the most prevalent non-viral sexually transmitted infection in the world. The objective of the study was to investigate the incidence of TV infection and its association with Human Papillomavirus (HPV) in a sample of Egyptian females.**

PATIENTS AND METHODS: 96 Egyptian females suspected for trichomoniasis were involved in our study. Vaginal washouts and cervical (cytobrush) samples were obtained from all patients and examined for *T. vaginalis* by direct wet mount, modified Diamond's culture medium, and real-time PCR. Cervical (cytobrush) samples were examined for HPV using real-time PCR.

RESULTS: Out of 96 patients, 28 (29%) was positive for *T. vaginalis* by real-time PCR. HPV was detected in 33 patients (34.4%); 31 cases (32.3%) were infected with HPV of genotype 16, whereas only two cases (2.1%) had genotype 18 infection. A significant association was found between TV and HPV infection [Odds ratio (OR)=10.58; 95% confidence interval (CI): 3.819 - 29.29; *p*<0.001].

CONCLUSIONS: When it comes to diagnosing trichomoniasis in a susceptible population, real-time PCR is more reliable than traditional standard approaches. A significant association between TV and HPV infection was found. Further research into the processes by which these two organisms interact at the cellular level could be revealed.

Key Words:
Trichomonas vaginalis, Human papillomavirus, PCR, Infection, Sexually transmitted infection.

Introduction

Trichomonas vaginalis infection is the most common non-viral sexually transmitted infection worldwide[1]. It remains a public health challenge due to high prevalence, and inefficient and inaccurate diagnosis, especially in developing countries[2]. Female patients infected with *T. vaginalis* have a wide range of clinical symptoms, ranging from asymptomatic to acute or chronic inflammatory disease. Trichomoniasis is accompanied by serious complications of the reproductive tract, including infertility, ectopic pregnancy, pelvic inflammatory diseases, premature rupture of membranes, preterm delivery, and low birth weight[3]. Additionally, it plays a significant role in human immunodeficiency virus (HIV) transmission and acquisition[1]. Human papillomavirus (HPV) is the most prevalent viral infection of the genital tract. There are more than 150 recognized strains of HPV; the majority of them are asymptomatic, but a few oncogenic kinds, mainly HPV-16 and HPV-18, are accompanied by a significant risk

Corresponding Author: Azza Mohamed Elamir, MD; e-mail: azzaelamer2013@gmail.com

of various anogenital malignancies, including cervical, vaginal, vulvar, and anal[4]. Numerous studies[5] have shown that women who resulted positive to *T. vaginalis* are four times more likely to have high-risk human papillomavirus. The inflammation caused by *T. vaginalis* leads to cervical epithelial disruption, which allows HPV to enter the basal layer of the epithelium, allowing viral DNA to be incorporated into the host DNA and increasing the expression of viral oncogenes, all of which participates in carcinogenesis process[6].

The aim of the current study was to investigate the prevalence of *Trichomonas vaginalis* infection in a sample of Egyptian females and its association with HPV.

Patients and Methods

We carried out an observational cross-sectional study included 96 female patients seen at the following hospitals in Egypt: Outpatient Clinics of Gynecology and Obstetrics; Fayoum University Hospital, Faculty of Medicine, Fayoum University and Saied Galal Hospital, Faculty of Medicine, Al-Azhar University.

Eligibility Criteria

We included all non-menstruating female patients with gynecological manifestations suggestive of trichomoniasis, including vaginal discharges with or without pruritis vulvae, dyspareunia, and dysuria.

Exclusion Criteria

History of intercourse or douching for the last 2 days, use of antiprotozoal or antibiotics for the last 2 weeks.

Each participant in the study provided a complete demographic and clinical history. A written informed consent was taken from each patient after brief description of the research idea. The study protocol has been reviewed and approved by the Ethical Committee at the Faculty of Medicine, Fayoum University (R-289). It was completed in accordance with the Ethical code of the World Medical Association (Helsinki's Declaration) for human-being experimentations.

Samples Collection

Vaginal washouts collection and cervical cytobrush samples were obtained from each participant[7] as follows:

- Sterile vaginal Cusco speculum was inserted into the patient's vagina then rotated 90° until an optimal view of cervix was seen.
- 5 ml of the sterile isotonic PBS solution was introduced into the vagina using sterile syringe without its needle.
- Fluid in the posterior fornix of the vagina was aspirated by the plastic pipette and put into sterile tubes. Additionally, cervical cytobrush sample was taken from the cervix and put into another tube.
- Vaginal washouts were examined for *Trichomonas vaginalis* by 3 methods: direct wet mount, modified Diamond's culture medium, and real-time PCR with TaqMan probe.

Cervical (cytobrush) samples were examined for Human papilloma virus using real-time PCR.

Direct Wet Mount Microscopic Examination[8]

Microscopic examination was done immediately after sample collection to detect motile flagellate *T. vaginalis* trophozoites using ×10, ×40 and ×100 objectives of a light microscope.

In Vitro Culture on Diamond's Tripticase-Yeast-Maltose (TYM) Medium

The inoculation of culture media was performed according to Garcia and Bruckner[9]. The vaginal washout was shacked then one drop was inoculated on the culture tube containing Diamond TYM medium and incubated at 37°C with daily microscopic examination for the presence of *T. vaginalis* trophozoites. Negative specimens were continually incubated under the same conditions until the seventh day following the initial inoculation, at which point samples with no trophozoites upon microscopic examination were considered negative for *T. vaginalis* using the culture method.

Molecular Study for Trichomonas Vaginalis

The DNA extraction

We used the PREP-NA-DNA mini kits [DNA-Technology, Catalog number: 378-2 (Moscow, Russia)].

(1) We added 300 µL of the lysis buffer into each tube at room temperature then we added 200 µL of the sample. (2) We added 400 µL of the precipitation buffer. (3) We vortexed the specimen suspension at maximum speed for 3-5 sec. (4) We placed the spin column filter in 2 ml

collection tube and applied the specimen suspension mixture to the spin column, incubated for 30 seconds and centrifuged at 13,000 rpm for 3 minutes. (5) We discarded the collection tube containing the filtrate solution and placed the spin column filter in a new 2 ml collection tube. (6) We added 500 µL of the wash-out solution 1 (W1), mixing by inverting the tube 3-5 times and centrifuged at 13,000 rpm for 2 minutes. (7) We discarded the collection tube containing the filtrate solution and added 300 µL of the wash-out solution (W2), mixing by inverting the tube 3-5 times, centrifuged at 13,000 rpm for 1 minutes, then, the filtrate solution was discarded, and we centrifuged the tubes at 13,000 rpm for 4 minutes. (8) We discarded the receiver tube, transferred the spin filter into dissolving tube, and pippeted 50 µL of elution buffer onto the membrane. (9) We incubated the tubes for 3 minutes at room temperature then centrifuged at 13,000 RPM for 1 minute to elute DNA. (10) This method gave a total DNA yield of about 30 µL, we used The NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) to determine the extracted DNA purity and concentration.

Real-time PCR amplification of T. vaginalis DNA (18S rRNA gene)

We used real time PCR detection kits for *T. vaginalis* [DNA-Technology, Catalog number: 234-5 (Moscow, Russia)].

(1) We mixed Taq-polymerase solution thoroughly (3-5 seconds), then spin briefly (1-3 seconds). (2) We added 10 µL of Taq-polymerase solution into the real time PCR Paraffin sealed tubes. (3) We added One drop (20 µL) of mineral oil into each tube. (4) We added 7 µL of DNA sample into the corresponding PCR-tubes. (5) We Loaded the plate into the real-time PCR system and running the plate [DTlite 4, DNA technology, (Moscow, Russia)].

The automatic analysis for Real-Time PCR using software downloaded from http://www.dna-technology.ru/eng/support/. Thermal profile for amplification and real time PCR detection of *Trichomonas vaginalis* is shown in Table I.

Detection of Human Papillomavirus

DNA extraction

We used GeneJET Genomic DNA Purification Kits [Thermo Fisher Scientific, Catalog number: K0721, (Waltham, MA, USA)], according to the manufacturer instructions.

(1) Collected cervical specimen was included in the cytobrush, so we put the cytobrush into a 2 mL microcentrifuge tube and resuspend in 180 µL of digestion solution. (2) We added 20 µL of Proteinase K Solution, mixed by vortex and incubated the sample at 56°C till the tissue is fully lysed for 30 minutes. (3) We added 20 µL of RNase A Solution, mixed by vortex then incubated for 10 minutes at room temperature. (4) We added 200 µL of lysis solution, mixed by vortex for 15 sec. (5) We added 4 µl of the internal extraction control DNA to each DNA sample once it has been resuspended in lysis buffer, then we added 400 µL of 50% ethanol and mixed by vortex. (6) We transferred the prepared lysate to purification column, inserted in a collection tube and centrifuged the column for 1 minute at 6,000 rpm. (7) We discarded the collection tube containing the flow-through solution. (8) We placed the GeneJET genomic DNA purification column into a new 2 mL collection tube and added 500 µL of Wash Buffer I, then centrifuged for 1 minute at 8,000 rpm. (9) We discarded the collection tube containing the flow-through solution, added 500 µL of Wash Buffer II (with ethanol added) to the purification column and centrifuged for 3 minutes at maximum speed. (10) We discarded the collection tube and transferred the DNA purification polumn to a sterile 1.5 ml microcentrifuge tube. (11) We added 200 µL of elution buffer to elute genomic DNA, incubated for 2 minutes at room temperature and centrifuged for 1 minute at 8,000 rpm. 12) We discarded the column, and the purified DNA was immediately ready for DNA amplification or stored at -20°C till used.

Amplification of E6 gene human papillomavirus 16

This was performed by the use of [Genesig® Advanced Kit, Primerdesign™, Catalogue number: Path-HPV16 (Chandler's Ford, England,)] to detect *E6* gene of human Papillomavirus 16.

Table I. Amplification program for detection of *T. vaginalis*.

	Temp.	Time	Number of cycles
1.	80.0°C 94.0°C	00:30 01:30	1
2.	94.0°C 64.0°C	00:30 00:15	5
3.	94.0°C 64.0°C	00:10 00:15	45
4.	94.0°C	00:05	1

Table II. Real-time PCR amplification protocol.

	Step	Time	Temp.
Cycling ×50	Enzyme activation Denaturation Data collection	2 min 10 s 60 s	95°C 95°C 60°C

Steps: We pipetted 15 µl of oasig™ 2X qPCR Master mix into individual wells of qPCR plate and added 5 µl of the extracted DNA. For negative control, we used 5 µl wells of RNase/DNase free water. The final volume in each well was 20 µl. PCR amplification protocol is shown in Table II.

Fluorogenic data was collected during this step through the FAM and VIC channels.

Device: step one real time PCR system (Thermo Scientific, Waltham, MA, USA).

Amplification of E6 gene human papillomavirus 18

Using Genesig® Advanced Kit [Primerdesign™, Catalogue number: Path-HPV18(Chandler's Ford, England)]. Amplification of *E6* gene human papillomavirus 18 was performed with the same steps done in amplification of *E6* gene human papillomavirus 16 protocol.

Statistical Analysis

The collected data were tabulated and analyzed using SPSS version 25 software (IBM Corp., Armonk, NY, USA). Data was presented as number and percentages. Chi-square test (X^2) or Fisher's exact test used as a test of significance. Confidence intervals for detection rate were determined using the student's *t*-test. Two-sided $p<0.05$ was considered significant.

Results

Detection of T. Vaginalis

Direct wet mount microscopic examination

Out of the 96 collected wash out vaginal samples, 8 (8.3%) were microscopically positive for *T. vaginalis* trophozoites (Figure 1).

Culture on Diamond TYM medium

Out of 96 vaginal washouts samples, 10 (10.4%) samples were positive for *T. vaginalis* after cultures (Figure 2). By cultures, eight samples out of these ten were previously proved to be positive by direct wet mount microscopic examination while the other two samples were negative by direct wet mount.

Detection of T. vaginalis by RT-PCR

Detection was made by using TaqMan based real time PCR, 28 out of 96 cases (29%) were positive for *T. vaginalis* infection, while 68 cases (71%) were negative (Figure 3). The 28 positive samples including the 10 positive samples identified by culture and another 18 positive samples.

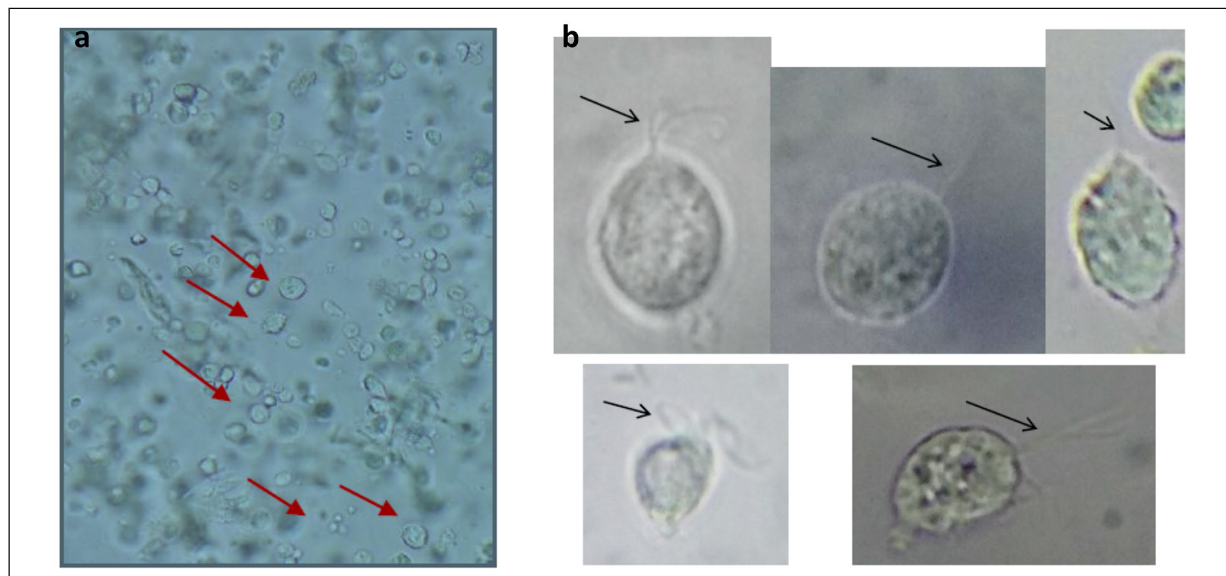
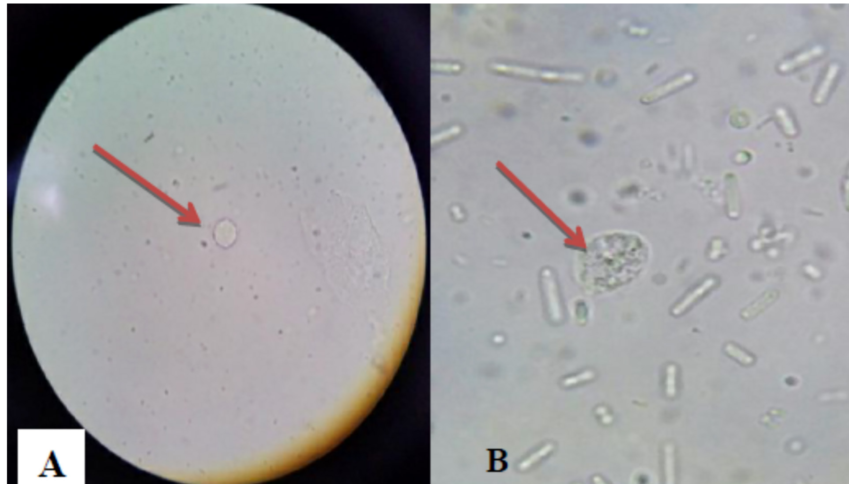


Figure 1. *T. vaginalis* trophozoite detected microscopically by vaginal wet mount examination using (**a**) high power (40×) and (**b**) oil immersion lens (100×).

Figure 2. *T. vaginalis* growth on TYM Diamond's medium detected by wet mount examination using oil immersion lens (100×). **A**, arrow points to round up *T. vaginalis* trophozoite; (**B**), arrow points to *T. vaginalis* trophozoite lost the typical pear shape. The *flagellae* were also invisible in both **A** and **B**.



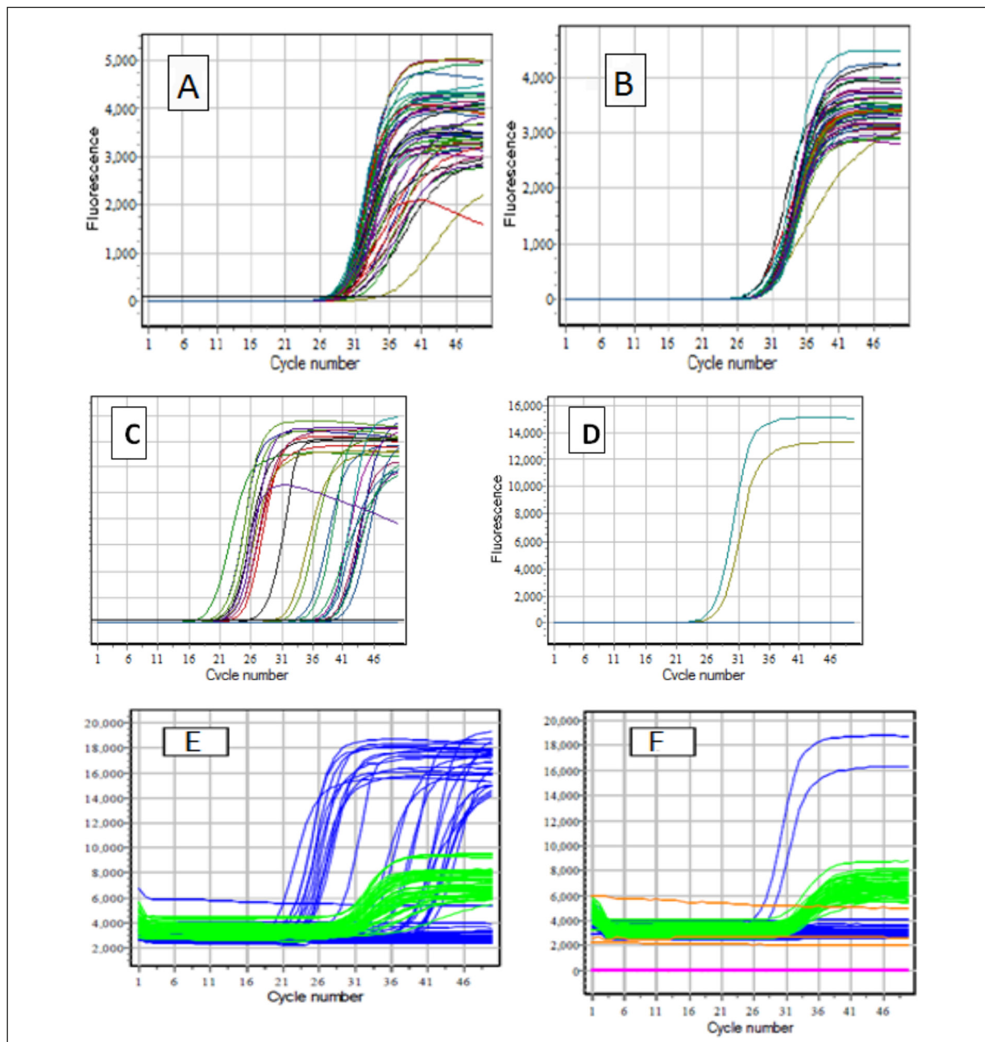


Figure 3. Real time PCR curves of the tested samples for *T. vaginalis*. **A-B,** Internal control included in each evaluated sample. **C-D**, show FAM channel fluorescence, in 26 positive samples (**C**) and other 2 positive samples (**D**). For (**E**) , and (**F**), the green curves represent the real time curves of HEX channel fluorescence for all of the tested samples (whether positive or negative), The blue curves represent FAM channel fluorescence and these are specific only for trichomonas positive samples. **E,** 26 Positive samples. **F,** 2 Positive samples.

Table III demonstrate that real time PCR attained the higher percent of positivity on detecting *T. vaginalis* infection (29%), followed by culture technique (10.4%), while the least percent of positivity was recorded by wet mount microscopic examination which detected only 8 cases (8.3%) with statistically significant difference between real time PCR compared to wet mount microscopic examination and culture on Diamond TYM medium with *p*-value < 0.001 and 0.00, respectively.

Detection of HPV

The infection with HPV was detected in 33/96 cases by percentage of 34.4% and the largest proportion of these cases; 31 cases (32.3%) were infected with HPV of genotype 16 and while the infection by HPV genotype 18 was detected in two cases only (2.1%) (Figure 4).

Table IV demonstrates that among 28 *T. vaginalis* infected cases there were coinfection with HPV in 20 cases representing (71.43%), while 8 cases (28.57%) were negative for HPV. There was a statistically significant association between *T. vaginalis* and HPV infections with Odds ratio (OR)=10.58; 95% confidence interval (CI): 3.819-29.29; *p*<0.001].

Discussion

Trichomonas vaginalis is an obligate extracellular flagellated anaerobic parasitic protozoan which causes trichomoniasis. Complications arising from this infection in women include preterm birth delivery, post-abortion infection and low-birth-weight babies. Besides, it may be a vehicle for genital viral tramsmissiom[2]. Poor diagnostic techniques for *T. vaginalis* also compounds the burden[10]. Rapid and accurate diagnosis of trichomoniasis is essential to administer appropriate treatment and to prevent the spread of the infection[11].

96 Egyptian females suspected for trichomoniasis were included in this study. In the present study, 8 samples (8.3%) were microscopically positive for *T. vaginalis* trophozoites. It is the simplest, rapid, and relatively cheap test but less sensitive. The parasite may be vanished if the sample is not examined within half an hour and may shape up in round form misleading with neutrophils[12]. Among Egyptian researchers, lower percent were recorded for positive *T. vaginalis* infection using direct wet mount in selected symptomatic females. These percents were 1% by Mahmoud et al[12], 1% by Hamdy and Hamdy[13] and 4% by Khalifa et al[14]. These researchers depended on vaginal swab or wooden spatula for taking the vaginal samples. Variant prevalence of *T. vaginalis* was recorded in different countries. In Iraq, Al Saeed[15] reported a prevalence of 2.4% among the collected vaginal swabs, while Hassan et al[16] reported 12.7% positive cases by wet microscopical examination of vaginal swabs, comparable to ours. Matini et al[17] in Iran, and Adjei et al[18] in Ghana, both recorded 1.7% of positive vaginal swab specimens by wet mount, and Leli et al[19] in Italy recorded 0.5% by microscopic examination.

In this study, 10 samples (10.4%) were found positive by culture on Diamond TYM medium. Many resources[20] recommended using Diamond TYM medium to improve the trichomonads detection in spite of its time consuming and late diagnosis of infection. Improvement of trichomonas detection by Diamond TYM medium culture in the present study is in agreement with Al Saeed[15] who recorded 5.4% using culture of *T. vaginalis* compared to 2.4% by wet mount. Also, the corresponding precents were 2.1% and 1.7% by Matini et al[17], 35.3% and 28.2% by Nassef et al[21], 3% and 1% by Mahmoud et al[12] and 6 % and 1% by Hamdy and Hamdy[13]. In contrary to the previous results is Khalifa et al[14] who detected 4% for both wet mount and culture on modified Diamond medium.

In our study, by using TaqMan-based real-time PCR, 18 positive samples were added to samples isolated by Diamond's culture raising the number to 28 positive samples.

Table III. Comparative prevalence of T. vaginalis infection among the studied cases according to diagnostic methods applied.

Test method	Positive No. (%)	Negative No. (%)	Chi-square test (X2)	*p*-value
Vaginal sample wet mount	8 (8.3)	88 (91.7)	13.675	< 0.001
Vaginal sample culture on TYM Diamond's medium	10 (10.4)	86 (89.6)	10.630	0.001
Real time PCR	28 (29)	68 (71)		

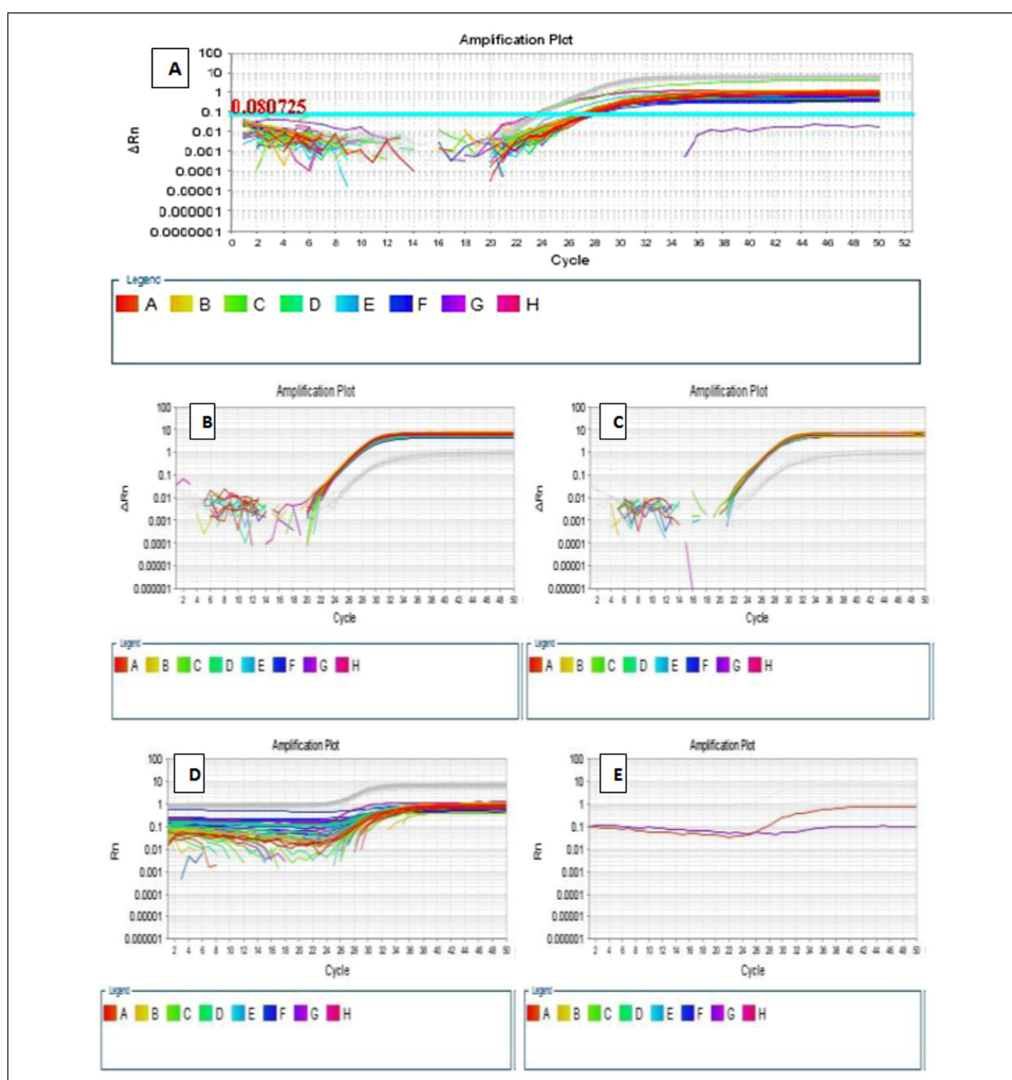


Figure 4. Real-time PCR curves of the tested samples for HPV. **A,** The internal control included in each sample for detection of HPV16. **B-C,** show FAM channel fluorescence which represent detected positive samples for HPV genotype16 in 31 samples (18 samples in B and 13 samples in C). **D-E,** show VIC channel fluorescence, which represent internal control for detection of HPV18 (**D**) and the detected two positive samples for HPV18 (**E**).

Table IV. Coinfection with *T. vaginalis* and HPV among studied cases upon using real time PCR.

	T. vaginalis								
Detected pathogen	**Positive**				**Negative**				**Total No. (%)**
	No. 28 + ve HPV		**% 29 - ve HPV**		**No. 68 + ve HPV**		**% 71 - ve HPV**		
HPV	**No. 20**	**% 71.43**	**No. 8**	**% 28.57**	**No. 13**	**% 20.63**	**No. 55**	**% 87.3**	**96 (100)**
Chi-square test (X^2)	24.058								
p-value	< 0.001								
OR (95% CI)	10.58 (3.819-29.29)								

Hassan et al[16] recorded that 100 samples (63.7%) were positive for *T. vaginalis* by P3 nested PCR. Depuydt et al[22] reported that the total prevalence of *T. vaginalis* in the general population in Flanders was 0.37% by using real-time PCR for diagnosis. In Russia, Shipitsyna et al[11] used 5 PCR assays to assess 448 samples (317 vaginal and 131 male urethral) among youth center attendees. The overall prevalence of *T. vaginalis* was 1.2%. In Italy, Leli et al[19] recorded 1.3% of positivity for *T. vaginalis* on using real-time PCR.

Among the complications that occur due to infection with *T. vaginalis* as a sexually transmitted parasite, are the pathogenic infections that accompanying the parasite; these pathogens include HIV[23], Herpes Simplex virus II[24], HPV and Chlamydia trachomatis[25,26].

In the present study, real-time PCR amplification was done to detect *E6* oncogene of human papillomavirus 16 and 18. Among all the studied cases 33 samples (34.4%) were positive for HPV and 63 samples (65.6%) were negative. 31 cases (32.3%) were the largest proportion of patients, infected with HPV of genotype 16, while the infection by HPV genotype 18 were detected in 2 cases (2.1%) only.

The rate of infection with HPV varied in different countries. Maher et al[27] in South Carolina reported that 50% of the studied population (HIV-infected women) to be high risk-HPV(HRHPV) positive as evaluated also by PCR while Camporiondo et al[28] recorded 24.9% in healthy Italian women using multiplex real-time PCR assays on cervical cytobrush samples of 309 healthy Italian women. Nearly double of our finding is that of Hackethal[29] in Brazil who recorded that by using multiplex PCR, 60% of women tested positive for HPV and HRHPV type 16 was most prevalent (47.2%).

HRHPV subtypes, particularly HPV-subtypes 16 and 18 are the main predisposing factors in most cases of cervical cancer[30].

In our study there is a statistically significant association between *T. vaginalis* and HPV infection, nearly ten-fold increased risk of HPV infection with Odds ratio (OR)=10.58; 95% confidence interval (CI), 3.819 - 29.29; $p<0.001$. Also, HPV serotype 16 was the predominant among *T. vaginalis* positive cases (93.9%), only one case was of serotype HPV 18 (6.1%). Like ours, Depuydt et al[22] by using Real-Time PCR found that prevalence of *T. vaginalis* to be higher in HPV infected women than in those without HPV infection (0.61 *vs.* 0.33%, $p < 0.0001$). This result is higher than the study of Maher et al[27] which showed that *T. vaginalis* infection was associated with a 4-fold increased risk of HRHPV infection (OR, 3.8; 95% CI, 1.6-9). Camporiondo et al[28] reported that *T. vaginalis* was found with a prevalence rate 1.3% of healthy Italian women and all of them were associated with HPV.

The hypothesized mechanism for increased risk of HPV coinfection and elevated risk of cervical dysplasia caused by HPV in case of *T. vaginalis* infection is the protozoan release inflammatory mediators causing cervical inflammation.

Inflammation disrupts the cervical epithelium, acting as a protective barrier, allowing HPV to penetrate to the basement membrane[31,32].

Because *T. vaginalis* can co-infect with viral and bacterial pathogens, Chigbu et al[33] suggested that presence of *T. vaginalis* in the lower genital tract may be utilized as an indicator for other more serious STIs caused by N. gonorrhoeae, Gardnerella vaginalis, C. trachomatis, and HIV. *T. vaginalis* may increase opportunistic virus entry into susceptible host cells by weakening the mechanical barrier of the vaginal epithelium. *T. vaginalis* secretes a cysteine protease which inhibits human secretory leukocyte protease inhibitor (SLPI) at the infected site and causes cell apoptosis[32]. Human SLPI is in charge of inhibiting other proteases released during an inflammatory response. In the absence of SLPI inhibition, inflammatory proteases can degrade surrounding tissue, allowing pathogens such as HIV and HR HPV to enter[34,35].

Conclusions

Wet mount microscopy and culture are undependable for detection of Trichomoniasis. We recommend a mandatory PCR test as a standard laboratory procedure for verification of negative results, considering that a negative wet mount does not exclude infection with *T. vaginalis*. We detected a significant association between TV and HPV infection, so we recommend a PCR assay for screening of HPV in recurrent cases of Trichomoniasis and more research into the processes by which these two organisms interact at the cellular level could be revealed.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding
This research did not receive any specific grant from external funding agencies. The study was completely sponsored by Fayoum University under supervision of its research committee.

Ethics Approval
The study protocol has been approved by the Ethical Committee at the Faculty of Medicine in Fayoum University, under the registration No. R-289.

Informed Consent
Consent was obtained from each patient.

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