How to Cite:
Rahi, A. A., & Jaleel, I. (2022). Feasibility of a nested PCR for the diagnosis of Trichomonas vaginalis infection in women a Wasit Province, Iraq. *International Journal of Health Sciences*, *6*(S8), 3490–3499. https://doi.org/10.53730/ijhs.v6nS8.12862

Feasibility of a nested PCR for the diagnosis of Trichomonas vaginalis infection in women a Wasit Province, Iraq

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***Abstract*---**This study was concerned the describe of an current PCR method for specific detection of *Tichomonas vaginalis* DNA into a rapid nested PCR assay, and to estimate the recent prevalence of *T. vaginalis* according to studying different factors that effect on vaginal trichomoniasis in women complaining of vaginal discharge. Vaginal swab samples were collected from 100 patients lived at Wasit province, during the period from January, 2022 to July, 2022. Demographic data, clinical and socioeconomic status were collected from the patients using a structured questionnaire. Total genomic DNA was isolated from 100 samples of vaginal swab randomly chosen to specific detection of *T. vaginalis* DNA into a rapid nested PCR. All samples were examined by the direct microscopic examination (wet mount). The vaginal swabs examined by wet mount and nested PCR to detect the presence of *T. vaginalis*. Of the 100 women, 29 were positive by nested PCR giving the prevalence rate of (29%) with and the direct microscopic examination (wet mount) showed 4/100 (4 %) positive case. Their ages were different. Women aged (20-30)years had significantly higher prevalence of trichomoniasis (14%) than other age groups. The high incidence of *T.vaginalis* infection was found among urban females (18%), followed by rural females (11%). Uneducated women had significantly higher rate of *T.vaginalis* infection (13%) than educated women (4%) . All infected women suffered from itching, 26 of positive case (29) suffered from vaginal discharge and 8 of them had dysuria.

***Keywords*---**Tichomonas vaginalis, nested-PCR, human, microscope.

Introduction

Trichomonas vaginalis can be define as a flagellated, an anaerobic protozoan parasite and the causative agent of trichomoniasis. This parasite can be transmitted by sexual intercourse from one host to another host [1]. Trichomoniasis act as the most prevalent non-viral sexually transmitted infection (STI) which affect human of all ages, ethnicity, and socioeconomic groups [2]. In the year 2012, the World Health Organization (WHO) evaluated that about 143 million new cases of *Trichomonas vaginalis* infection were recorded in adults aged from 15 to 49 years [3]. *Trichomonas vaginalis* can infect both females and males; but, in women, the rate of infection is much higher [4]. Pregnant women infected with *T. vaginalis* have a great risk of preterm delivery because *T. vaginalis* lead to the membranes premature rupture. A previous research has shown that pregnant women infected with *T. vaginalis* have about 30% risk of preterm delivery or delivering infants who have a low birth weight [5]. *Trichomonas vaginalis* infection also aid to the increase in HIV transmission from mother-to-child [6].

In spite of the clinical symptoms of *T. vaginalis* infection, there are many questions regarding its pathogenicity, epidemiology, and drug resistance yet. Studies to answer many of these questions would be greatly helped by a definite classification method for *T. vaginalis*. Commonly, PCR and its related technique, as a reliable and sensitive methods, were broadly used for genetic research in molecular epidemiology of organisms [7].

Materials and Methods

Direct microscopic examination

Vaginal swab were collected from 100 women lived at Wasit Province during the period from January, 2022 to July, 2022. All participants with symptoms, after speculum enrollment, one vaginal swab were taken from each patient, these swabs were used to obtain direct microscopic examination by wet mount method which attained by mixing of the vaginal fluid with a normal saline drop and directly examined by microscope at 10 x and 40 x to observe the movement of trophozoite [8]. Then, the specimen were kept in -20 °C for molecular detection. Moreover, information on the following aspects were also obtained from each patient subject: age, residency, level of education, pregnancy and signs and symptoms.

Molecular detection by Nested- PCR

The Nested PCR technique was performed for detection *Trichomonas vaginalis* based on conserved region in small subunit ribosomal RNA gene from vaginal swab samples. This method was carried out according to [9] which involved genomic DNA extraction. Genomic DNA was extracted from vaginal transport media swab samples by using G-spinTM Total DNA Extraction Kit (Body fluid protocol).Then , the extracted genomic DNA was checked by using nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).The third step was

nested PCR master mix preparation .The PCR master mix was prepared using (GoTaqTM Green PCR Master Mix) and this master mix done according to company instructions as following tables:

First round PCR master mix

PCR Master mix	Volume
DNA template 5-50ng	5µL
Trichomonas vaginalis small subunit ribosomal RNA gene PCR Forward primer (10pmol)	2µL
Trichomonas vaginalis small subunit ribosomal RNA gene PCR Reverse primer (10pmol)	2µL
Green PCR Master mix	12.5µL
Nuclease Free water	3.5 µL
Total volume	25µL

After that, these PCR master mix component that mentioned in table above transferred into Exispin vortex centrifuge at 3000 rpm for 3 minutes. Then placed in PCR thermocycler. The PCR thermocycler conditions by using convential PCR thermocycler system. The second round PCR master mix was the following step.

Second round PCR master mix

PCR Master mix	Volume
First round PCR product	2.5µL
Trichomonas vaginalis small subunit ribosomal RNA gene Nested PCR Forward primer (10pmol)	2µL
Trichomonas vaginalis small subunit ribosomal RNA gene Nested PCR Reverse primer (10pmol)	2µL
Green PCR Master mix	12.5µL
Nuclease Free water	6 µL
Total volume	25µL

After that, these Nested PCR master mix component that mentioned in table above transferred into Exispin vortex centrifuge at 3000 rpm for 3 minutes. Then placed in PCR Thermocycler.

Nested PCR product analysis

The Nested PCR products of was analyzed by agarose gel electrophoresis . 2% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C. Then 3µl of ethidium bromide stain were added into agarose gel solution. Agarose gel solution was poured in tray after

fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray. The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. 10µl of Nested PCR product were added in to each comb well and 3µl of (100bp Ladder) in first well.Then electric current was performed at 85 volt and 80 mA for 1.5hour.The Nested PCR products were visualized by using UV Transilluminator.

Results

In the present study, sensitivity of the nested PCR was tested. Different amplified DNA fragments of *T.vaginalis* were obtained from the reactions between both forward primers with the reverse primers and the PCR products were visualized as ethidium bromide- stained bands of 524 bp resolved by agarose gel electrophoresis. Negative controls did not show any reactions. The reaction Semi quantification of *T. vaginalis* was done after serial dilution of the DNA samples (Figure :1and 2).

On the other hand, wet mount was read for all the received samples from the total of 100 women that were enrolled in this study with signs and symptoms of vaginitis. Results of wet mount and PCR test were compared, as summarized in Table 1. The rates of *T. vaginalis* infection were calculated by the both methods. Only 4 (4%) positive samples were diagnosed by wet microscopy. Regarding nested PCR, positive reaction was observed in 29 samples, which confirms that PCR assay was more sensitive than wet preparation microscopy.

The results of the current study were showed that the highest incidence of *T.vaginalis* infection occurs in age group (20-30) years with the percentage of (3%) and (14%) detected by direct smear and nested PCR, respectively, followed by the age group (31- 40) years with the percentage of (1%) and (12%) detected by direct smear and nested PCR, respectively, as seen in table (1). Table (2) shows *T.vaginalis* infection rate in relation to residency of patient involved in the study, high rate of *T.vaginalis* trophozoites were recorded in vaginal discharges from urban area (the center of Wasit province) 3% and 18% detected by direct smear and nested PCR, respectively compare to 1% and 11% in samples of patient from rural area by direct smear and nested PCR, respectively .

In the current study, the incidence of *T. vaginalis* infection was the highest in the women with low education level which had the percentage of (2%) and (13%) by direct and nested PCR, respectively. and the lowest incidence of infection was in women who have high education level , which had the percentage of 1% and 4% respectively, as shown in table (3). In the existing results, vaginal discharge, itching (3%, 4% respectively) by direct microscopic examination and (26% , 29% respectively) by nested PCR detection followed by dysuria 1% and 8% respectively, by direct and indirect methods were the main symptoms in participants infected with *T. vaginalis* as shown in table (4).

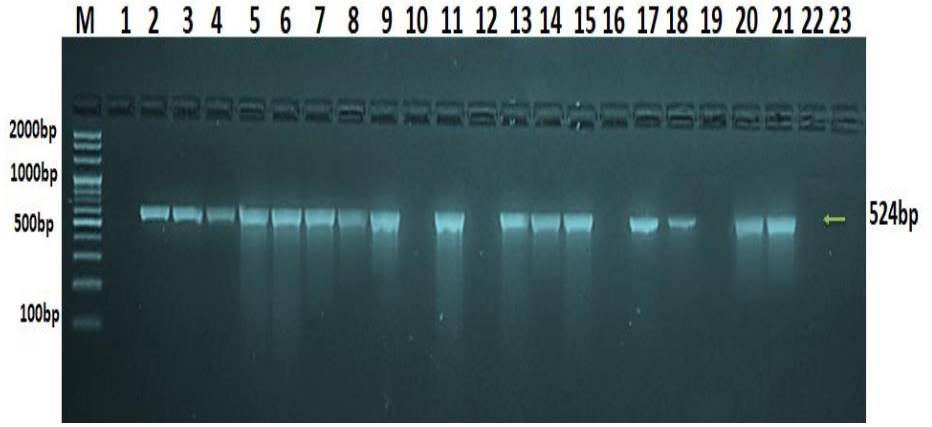

Figure 1: Agarose gel electrophoresis image that showed the Nested PCR product analysis of conserved region in small subunit ribosomal RNA gene in *T.vaginalis* from vaginal swab samples. Where M: marker (2000-100 bp). Lanes (1-23) showed some Positive *T. vaginalis* samples at (524 bp) Nested PCR product.

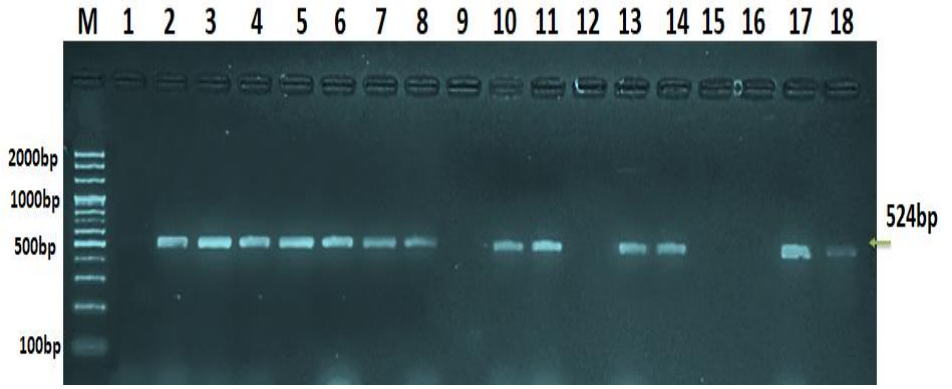

Figure 2: Agarose gel electrophoresis image that showed the Nested PCR product analysis of conserved region in small subunit ribosomal RNA gene in *T. vaginalis* from vaginal swab samples. Where M: marker (2000-100 bp). Lanes (1-18) showed some Positive *T. vaginalis* samples at (524 bp) Nested PCR product.

Table (1): Incidence of *T. vaginalis* detection by direct smear methods and Nested PCR according to age

Age Group/year	Total No. Examined	(%)	Direct smear examination + Ve	(%)	Nested PCR + Ve	(%)
< 20	9	9 %	0	0 %	1	1 %
20-30	35	35 %	3	3 %	14	14 %
31- 40	33	33 %	1	1 %	12	12 %
41- 50	15	15 %	0	0 %	2	2 %
>51	8	8 %	0	0 %	0	0 %
Total	100	100%	4	4 %	29	29 %

Table(2): Incidence of *T. vaginalis* detection by direct smear methods and Nested PCR according to residence

Residency	Total No. Examined	(%)	Direct smear examination + Ve	(%)	Nested PCR + Ve	**(%)**
Rural	59	59%	1	1%	11	11%
Urban	41	41%	3	3%	18	18%
Total	100	100%	4	4%	29	29%

Table(3): Distribution of *T.vaginalis* infection detected by direct smear methods and Nested PCR according to level of education

Level of education	Total No. Examined	(%)	Direct smear examination + Ve	(%)	Nested PCR + Ve	(%)
High	17	17%	1	1%	4	4%
Moderate	39	39%	1	1%	12	12%
Low	44	44%	2	2%	13	13%
Total	100	100%	4	4%	29	29%

Table (4): Incidence of *T. vaginalis* detection by direct smear methods and Nested PCR according to signs and symptoms

	Vaginal discharge	Itching	Dysuria
Total No.	100	100	100
Frequent No.	86	81	32
Direct examination + Ve	3	4	1
Nested PCR + Ve	26	29	8

Discussion

Definitive diagnosis of *T. vaginalis* is affected by many factors, including patient factors, specimen sampling, clinician's experience, processing and test version, skill set and experience of those doing microscopic examination. Furthermore, specificity, sensitivity, ease of use, cost and time to results for the different methods for diagnosing infection of *T.vaginalis* should be also considered [10]. In the present study, 100 vaginal samples were examined by wet mount and nested PCR. whereas, 29 positive cases of *T. vaginalis* were detected. The nested PCR was able to diagnose the 25 cases missed by wet mount method. However, direct wet-mount microscopic examination is the most available and widely used in clinical practice, as it is cheap and easy to do. However, *T. vaginalis* able to produce an over-whelming inflammatory response, concealing the parasites, or the very low number of organisms. Thus, even with skilled diagnosticians, the diagnostic sensitivity of wet-mount method is only 60% compared with another methods [11].

From the other hand, molecular diagnosis of *T. vaginalis* was first invention by [12] in clinical samples using primers TVA6 and TVA5. Subsequently, many additional primer sets have been described. The sensitivity and specificity of these primers in clinical studies using vaginal swab specimens have varied, with

sensitivities of 85% to 100% being reported. The technique's sensitivity was such high as to be able to amplify and detect just one trichomonad and which contains as low as 0.15 pg of DNA, which is 100-fold less than DNA/host cell [13].One factor that helps increasing the sensitivity of this PCR is that the copy number of the Tv-E650 repeat has been estimated to be about 100 to 1000 repeats /genome [14]. The present results are in agreement with[15], in which their PCR assay was able to detect as few as five *T. vaginalis* organisms per millilitre of medium.

Among the main factors that contribute to low test sensitivity with wet-mount microscopy may be the use of dry swabs or delayed transportation of the specimen to the laboratory. The low sensitivity of wet-mount microscopy makes it unsuitable as the only test for laboratory diagnosis of trichomoniasis. This study confirms the results of others [16], in showing that PCR testing was more sensitive than culture for the detection of *T. vaginalis*.

Several PCR assays targeting different regions of the *T. vaginalis* genome have been described for diagnosis of this infection [17]. These studies showed a sensitivity by PCR in the range of 89–98 %. The high prevalence of this sexually transmitted infection probably results from the increased detection sensitivity of PCR compared with wet-mount microscopy. The high sensitivity and specificity of PCR reported in this study would offer a useful rapid screening tool. This could reduce spread and transmission of the infection, in particular from asymptomatic patients.

The two age groups (20-30)years and (31-40) years have higher incidence with this infection than the age (< 20) years , (> 41) years. This result can be explained by the fact that incidence with this parasite may be related with age group of menstrual cycle .This result agreed with explanation of some authors [18]. So, age groups (>41) year showed that the lowest incidence with this parasite infection, and these results disagreed with the study of [19].This occur due to the ability of the parasite to alternate the vaginal environment for its survival. A study done by [20], showed that the highest infection rate (80%) was found in women of reproductive age (25-45) years, and 9% were aged less than 25 years; and 30% were above 45 years of age.

The lowest incidence was found among age group (44-63) years, and this is probably due to the absence of suitable environment for growth of *T.vaginalis*. Studies thus it is believed that the incidence of *T.vaginalis* infection increases proportionally at the reproductive age and then decline, depending on several factors including mainly hormonal factors (Pregnancy, aging etc) and sexual activities. Also, the pH begins to fluctuate back and forth causing an imbalance [21].

The results of our study showed that high rate of *T.vaginalis* trophozoites were recorded from urban area (the center of Wasit province) 3% and 18% detected by direct smear and nested PCR, respectively. This result disagreed with the report by [22], who reported that the peak of incidence of disease in patients who were living in rural area. In the present study, high percentage of infection was seen in females with the uneducated women from women who have education but

this is not acceptable since most of these women disagree to do gynaecological examination, This result is in agreement with [23].

The low rate of infection among women with social and educational advantage may be due to their better hygienic habits, and since they are keener to ask for medical advice than indigents who may accept vaginal discharge and minor irritation as being normal. The results of the present study showed that *T.vaginalis* infection may be accompanied with different clinical symptoms, discharge with itching, discharge with itching and dysuria , discharge only and discharge with dysuria. This symptoms actually resulted due to the parasites invasion to genital and urinary system. *T. vaginalis* grows best at alkaline environment and attaches itself by its axostyle thus creating the irritation, inflammation, and other symptoms associated with the infection by this parasite [24]. Although our result agreed with those of [25] who found that *T.vaginalis* may be found in any type of vaginal discharge .

Conclusions and Recommendations

Sensitivity of nested PCR for *T.vaginalis* using vaginal swab sample exceed the direct wet mount examination. Additionally, the cost of nested PCR highly exceeds the direct wet method .Present study contributed in giving more data on prevalence of *T.vaginalis* infection among women in Wasit Province in Iraq. The present study also detected some important factors related to infection and transmission of disease and referred to the need for more study in this problem. Accurate diagnosis will always result in better control and suitable treatment of *T.vaginalis* .The molecular methods and traditional laboratory methods should be used side by side with each other for a rapid and a perfect detection of *T.vaginalis* infection of women. Additional study is required to evolve and expand sensitive and cheap diagnostic tests, allowing better understanding of the epidemiology of *T. vaginalis* and targeted inspection of asymptomatic individuals.

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