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Evaluation of wet mount, Rapid antigen test and molecular techniques in the diagnosis of *Trichomonas vaginalis* in men

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Abstract: *Trichonas vaginalis is a sexual, human flagellated protozoan-obligated parasite. In Iraq, there are few studies concerning trichomoniasis in men and no studies were conducted on men in Maysan province. So this study was conducted to estimate the infection rate (IR) of T. vaginalis in males and evaluated four methods that used in diagnosis of this parasite including Wet mount, Rapid antigen detection test (RADT), Conventional polymerase chain reaction (CO-PCR), and Real-time quantitative polymerase chain reaction (RT-qPCR). Urine and semen samples were collected from 97 human males whose ages ranged from 18 to 50 years, and agreed to participate in the current study.*

With the wet mount (WM), no one had been detected to be infected (0/97) with T. vaginalis. In RADT, it finds that 13.63%(6/44) of urine samples are positive for trichomoniasis by this test while all the semen samples gave negative results. Conventional PCR detected T. vaginalis in 18.18% (8/44) of urine samples and in 27.27% (12/44) of the semen sample. In Real-time quantitative PCR (RT-qPCR), T. vaginalis was detected in 29.54% (13/44) of urine samples and 31.82% (14/44) among the semen of the same males. The WM had the lower sensitivity of 0.0% and RT-qPCR had the highest sensitivity (100%) Finally, it can be concluded that the RT-qPCR is a golden standard tool for the diagnosis of T. vaginalis with high sensitivity and specificity and wet mount was poor and not recommended.

.**Keywords:** T. vaginalis, males, wet mount, RADT, RT-qPCR, PPV

1.Introduction

T. vaginalis Donné, 1836 (Family: Trichomonadidae, Wenyon, 1926, Class: Parabasaila, Margulis, 1990) is an anaerobic extracellular flagellated protozoan obligated parasite in humans only, it had been observed at the first time in vaginal secretion by Alfred Francois Donné in 1836 (Donné, 1836; Soper, 2003).

T. vaginalis infected humans only and no vector or reservoir hosts in its life cycle (Kusdian and Gould, 2014). It takes an oval or pear shape, and sometimes, it even resembles an amoebal shape (Mahmud *et al*., 2017). It infected the urogenital tract of both human genders, male and female at all ages, adults, and children, in all regions of the world, whether they were rich or poor (Morris et al., 2021). WHO reported that 276.4 million cases in 2008 are infected with *T. vaginalis* 90% of them located in poor regions (Rowley *et al*., 2019). Symptoms of the disease may occur in the infected individual during the period of incubation, which is between (5-28) days, but some individuals do not suffer from any symptoms (Tompkins *et al*., 2020). In males, Trichomoniasis is frequently asymptomatic or causes urethritis, which is often linked with cystitis, prostatitis, and epididymitis (Dalimi and Payameni, 2021). Some semen characteristics are affected by *T. vaginalis* infection (Sena *et al*., 2007), as reducing the motility, viability, morphology, and increased seminal fluid viscosity (Gopalkrishnan *et al*., 1990). It showed that the infertility was induced by infected with *T. vaginalis* which is usually related to biophysical damage in spermatocytes. This damage associated with intensity of *T. vaginalis* in seminal fluid (Martinez *et al*., 1996).

WHO reported that the globally *T. vaginalis* IR among males was estimated to be 1.0% (WHO, 2001), and about 0.5% in the USA, 0.6% in Europe and 0.1% in Asia (WHO, 2012).

In some Iraqi provinces, it showed that the IR of male trichomoniasis in Basrah was 12% (Khalaf *et al*., 2010), Erbil, 4.84% (Al-Jadoa and Mawlood, 2010), Al-Najaf, 17.64% (Al-Kafagy *et al*., 2014), and Babylon, 9.29% (Al–Quraishi, 2015).

In males, the accuracy of *T. vaginalis* diagnosis depends on the test method and the type of sample, urine, sperm, or semen fluid. Many methods are used in diagnosis of *T. vaginalis* including, microscopic, cultural, immunological, and molecular methods (Bruni *et al*., 2019). A wet mount (WM) is a widely used method because it's simple, easy, and cheap. Asmah *et al*. (2018) found that its sensitivity and specificity were 31.6% and 100% respectively. The WM sensitivity is depended on the examiner's experience, the sample collection period, the time between sample collection and examination, and the parasite intensity (>10^4 organisms/ml) (Garber, 2005; Patil *et al*., 2012; Hobbs *et al*., 2013). The RADT is based on immunochromatographic capillary flow and the presence of polyclonal antibodies in the buffer (Meites *et al*., 2015). The advantage of this test is easy and rapid (takes about 30 min), safe (as a compact strip), and has low sensitivity and high specificity reach of 37.5% and 82.9 % respectively (Sheele *et al*., 2018). In conventional PCR (CO-PCR) the sensitivity and specificity reach 100 % and 99.9 % respectively (Khalaf *et al*., 2010). The Real-time quantitative PCR (RT-qPCR) had a high sensitivity of 100 % and high specificity of 99.6 % (Caliendo *et al*., 2005).

This study aimed to estimate the IR of *T.*

vaginalis among men in Maysan province with using some methods including direct microscopy/WM, immunological/RADT, and molecular such as CO-PCR and R-qPCR to detect *T. vaginalis* in urine and semen samples, and compare between these methods.

2.Methodology

The Ethical approval of the current study was obtained from the Maysan HealthDirectorate, Ethical Review Board with reference No. 2178 on 29/11/2021.

All precipitates who delivered samples and accompanying data were de-identified and allotted arbitrary numbers to ensure anonymity

In **WM,** 0.5 ml of each urine or semen was taken aseptically, and centrifuged at 3000 rpm for 5 minutes, then three slides were prepared from each specimen and examined under the light microscope (40x), and recorded the results of examination according to see the *T. vaginalis* or not.

In **the RADT test**, one cassette and one dilution bottle containing buffer were used for each sample. A half ml of each semen or urine sample was added into the dilution buffer bottle and mixed well for 60 seconds. four drops of the mixture were added in the cassette sample well. The result was read after 15-30 minutes. When the line of antigen- antibody reaction is appeared, this indicated as positive result.

CO-PCR, the *T. vaginalis* DNA was extracted using the Quick-DNA Miniprep kit (Zymo Research, USA), the extraction method was done according to the instruction of the kit manufacturing company. The purity and the concentration of the extracted samples DNA were estimated by A Nanodrop.

A specific primer of the *β-tubulin* genes, Forward 5′-TCCGTGGCCGTATGTCATCT- 3′ and Reverse 3′-GCTGTTGTGTTGCCGATGAA-5′ (169 bp, Alpha DNA Canada, USA) (dos Santos *et al*., 2015). In each PCR reaction-microtube 25µl were taken as a final volume consisting of 5µl of Taq polymerase, 1µl of each forward and reverse primers (10 ρmol/µl), 3µl of the DNA sample, and 15µl of DW, and the PCR conditions were used as designated by dos Santos et al. (2015) with some optimizations as follows: initial denaturation-1 cycle at 95º C for 5 minutes, 40 cycles included: denaturation-2 at 94º C for 45 seconds, annealing at 63º C for 45 seconds, extension-1 at 72º C for 45 seconds and finally, extension-2, 1 cycle at 72º C for 10 minutes.

The amplification products of DNA fragments were run through a 1.5% agarose gel in TBE buffer, using a 100bp ladder, in an electrophoresis unit (CBS, Scientific, USA) for 90 min at 70V (Sambrook *et al*., 1989). After running, the gel was stained by immersing it in a container containing RedSafeTM Nucleic Acid Staining Solution 20,000x (Intron, Korea), 3µl in 500ml DW, then it was photographed by gel document system (Labnet, USA).

RT-qPCR, DNA was extracted by using the PrimeScriptTM RT reagent Kit (TaKaRa, Japan), Maxime PCR PreMix kit (iTaq) (Intron, Korea), KAPA SYBR® FAST qPCR Master Mix (2X) Kit (KAPA, USA), and RNA extraction (Direct-zol™ RNA MiniPrep) (Zymo Research, USA). The extraction method was carried out according to the instruction of the kit manufacturing company. The same specific *β-tubulin* gene primers which used in CO-PCR are used in this test (dos Santos *et al*., 2015) with some optimizations as follows: pre-

denaturation at 95º for 5 min as hold cycle, then 40 cycles each consisted of denaturation at 95º for 2 min, annealing at 62º for 2 min, extension 72ºfor 2 min.

Statistical analysis:

The statistical analysis of this study data was done by SPSS (version 28) software using Chi-square (χ^2), test and t-test and used a probability value $p \leq 0.05$ as statistically significant criterion to the evaluation of wet mount, rapid antigen detection test, and molecular techniques in the diagnosis of *T. vaginalis* in males.

RESULTS

The results of this study (Table 1) showed that the direct microscopy examination of the wet mount of urine and semen samples of 97 participants males could not detect any infection with *T. vaginalis* (IR was 0% for each semen and urine).

The use of the RADT technique (Figure 1) for 44 randomly selected males showed (Table 1) that the *T. vaginalis* IR was 13.63% (6/44) of urine samples whilst it could not detect any infection in semen samples (IR=0.0%). Additionally, of the of urine samples, it showed that this test had a sensitivity 35.7%, a specificity 96.7% and an accuracy 77.3% .

Table 1 : A comparative between different diagnostic methods for detecting *T. vaginalis* among males in Maysan province

Method	sample	No exam	TP	FP	∑P	IN %	TN	FN	∑N	SE %	SP%	PPV %	NPV %	Acc %
WM	Urine	97	UD	UD	UD	UD	83	14	97	UD	100	-----	86.2	68.2
	Semen	97	UD	UD	UD	UD	83	14	97	UD	100	-----	68.2	68.2
RADT	Urine	44	5	1	6	13.63	29	9	38	35.7	96.7	83.3	76.3	77.3
	Semen	44	UD	UD	UD	UD	30	14	44	UD	100	-----	68.2	
CO-PCR	Urine	44	8	0	8	18.18	30	6	36	57.1	100	100	83.3	86.4
	Semen	44	12	0	12	27.27	30	2	32	85.7	100	100	93.8	95.5
RT-qPCR	Urine	44	13	0	13	29.54	30	1	31	92.8	100	100	96.8	97.7
	Semen	44	14	0	14	31.82	30	0	30	100	100	100	100	100

TP: True positive, FP: False positive, ∑p: Summation negative, SE: Sensitivity. Sp: specificity, PPV: Positive predictive value, NPV: Negative predictive value and Acc: Accuracy

The CO-PCR detected about 18.18% (8/44) of urine and 27.27% (12/44) of semen samples, it showed that this test had a sensitivity 57.1%, a specificity 100% and an accuracy 86.4%, and of the semen samples, it had a sensitivity 85.7%, a specificity 100% and an accuracy 95.5% (Table 1).

Of the 44 males, the RT-qPCR technique detected that 29.54%(13/44) of urine and 31.82% (14/44) of semen samples were positive for *T. vaginalis* (Table 1 and Figure 3). This study (Table 1, Figure 3) shows that the RT-qPCR had the highest accuracy for detecting T. vaginalis in the semen (100%) and urine (97.7%) and the highest sensitivity (100% and 92.8%) and specificity (100% and 100%) for detecting T. vaginalis in semen and urine respectively.

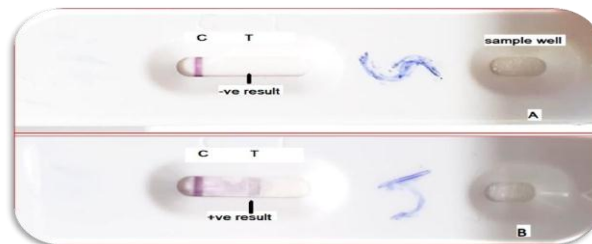

Figure 1: The cassettes of rapid antigen detect test (RADT) which using to diagnosis *T. vaginalis* A: positive result of urine sample, and B: negative result of semen sample.

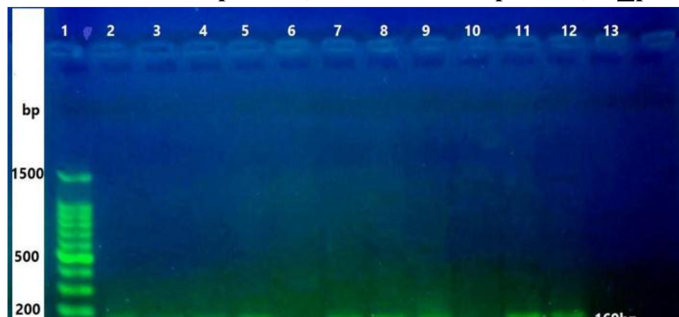

Figure 2: 1.5% agarose gel electrophoresis of the *T. vaginalis* β-tubulin gene CO-PCR DNA product t size 169 bp. Lines, 1:100 bp DNA Ladder; 2-5, 7-9,11: *T. vaginalis* positive cases, 6: *T. vaginalis* negative case, 12: positive control, 13: negative control.

However, the cycle threshold value (Ct value) of all positive samples of urine and semen occurred between the cycles 19 and 21 (Figure 3A). In another hand, it showed that the peaks of PCR reactions of all positive samples of both urine and semen occurred at the temperature ranged between 75 to 78˚C (Figure 3B). Additionally, It showed that *β-tubulin* gene of each sample DNA had a single peak.

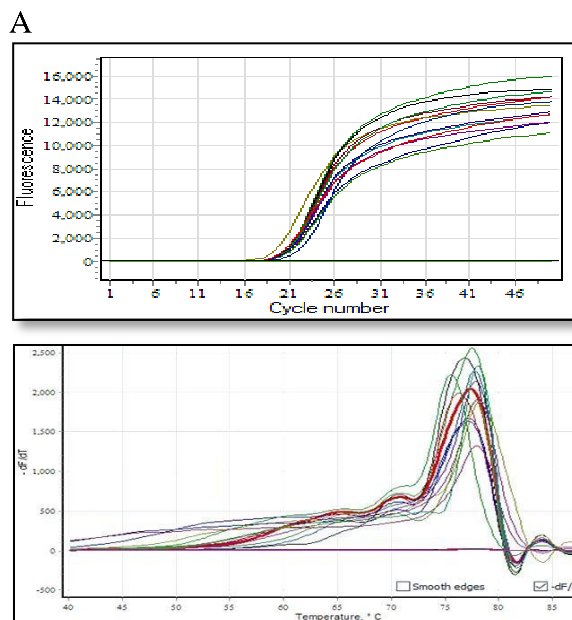

Figure 3: The RT-qPCR amplification curve of the *T. vaginalis* β-tubulin gene, A: the cycle threshold (Ct) of each positive samples and B: the curve of optimum temperature.

The statistical analysis of this study data showed significant differences ($\chi2=50.355$, $p<0.001$) between the four diagnostic techniques, wet mount test (WM), rapid antigen detection test (RADT), conventional PCR (CO-PCR), and real-time quantitative PCR (RT-qPCR) in their ability for detecting *T. vaginalis* in the urine and semen of the males.

DISCUSSION

The infection of males with the parasite *T. vaginalis* is not given as much attention as is the case with females, this may be due to the lack of clear symptoms in a high percentage of men compared to women. To date, most trichomoniasis research has been conducted in women (Van Gerwen et al., 2021). So, this study is conducted to investigate the IR of males whose ages ranged from 18 to 50 years. Four diagnostic techniques, including wet mount test (WM), rapid antigen detection test (RADT), conventional PCR (CO-PCR), and real-time quantitative PCR (RT-qPCR) are used to identify their ability to detect *T. vaginalis* in the urine and semen of the males.

This study (Table 1) shows that the MW/direct microscopy failed to detect *T. vaginalis* in any sample of the 97 urine and semen samples. MW had the lowest sensitivity (0.0%) and the lowest accuracy (68.2%). These findings are consistent with the findings of Gaydos et al. (2017), Asmah et al. (2018), and Tchankoni et al. (2021) that this method had low sensitivity in detecting *T. vaginalis*. The low sensitivity of the MW/direct microscopy method in detecting the parasite is attributed to several reasons, such as the movement of trophozoites in a wet mount, light infection, low intensity of *T. vaginalis*, history of treatment, and the lack of experience of the microscopic examiner

especially since it relies on seeing the parasite moving (Nye et al., 2009). For this, it can be said that this method was not recommended in the diagnosis of *T. vaginalis* (Rogers *et al*., 2014).

With regard to the RADT method, this study showed the trichomoniasis IR in the urogenital tract of the males was 13.63% in urine samples. This finding is higher than that found by Tompkins *et al*. (2020) who found that the IR of this test among males was 0.5% and less than that found by Gaydos *et al*. (2017) who found the IR was 23.4%. The RADT (Table 1) detected only 35.7% (5/14) of the total positive infections but could not detected any infection in semen. RADT has a low sensitivity of 0.0, and 35.7% among semen and urine samples respectively, and it has a high specificity of 96.7% and 100% respectively with accuracy of 77.3% and 68.2% respectively. These results agree with the findings of Asmah *et al.* (2018) and Sheele *et al*. (2019) who found that RADT had low sensitivity (25% and 37.5% respectively) and high specificity (83.3% and 82.9% respectively).

Some find that the PCR techniques are the golden methods for detecting *T. vaginalis* in the urine and semen of males (Asmah *et al*., 2018). And showed that the Positive Predictive Value (PPV) and Negative Predictive Value (NPV) are very high in RT- qPCR compared to other methods .

Positive predictive value (PPV) and negative predictive value (NPV) are the proportions of positive and negative outcomes on statistical and diagnostic tests that represent true positive and negative outcomes, respectively. They describe the performance of a diagnostic test or other statistical measure. PPV and NPV use the prevalence of a condition to determine the likelihood of a test diagnosing that specific disease, while sensitivity and specificity are independent of prevalence (Altman and Bland, 1994).

The current results showed that the IR of trichomoniasis using CO-PCR technique was 18.18% among urine samples of participants males and 27.27% among semen samples. These results were higher than those by Zhang *et al*. (2022) who revealed that CO-PCR detected 7.8% and 2.97% of urine and semen samples respectively and less than that recorded by Hobbs *et al*. (2006) of pointed that the CO-PCR detected *T. vaginalis* in 53.8% of urine and 69.3% of semen respectively. This study shows that CO-PCR had moderate sensitivity (57.1%) and high specificity (100%) for urine samples and had high sensitivity (85.7%) and specificity (100%) for detecting *T. vaginalis* in semen samples and had high accuracy 95.5% and 86.4% respectively. These results of urine are in line with what Asmah *et al*. (2018) found a sensitivity of 40% and a specificity of 100% respectively, and in semen with Van Gerwen *et al*. (2021) who found a high sensitivity (97.2%), and specificity (99.9%) and with Zhang et al. (2022) who found that the sensitivity and specificity were 99.7% and 100% respectively.

The RT-qPCR technique finds that the IR of *T. vaginalis* was 29.54% among urine samples of the participant males and 31.82% among semen samples. This finding is similar to finding by Simpson *et al.* (2007) who revealed that the sensitivity of RT- qPCR for detecting *T. vaginalis* in urogenital samples was 96% and specificity 100%.

Depending on the type of PCR technique, it is capable of detecting a very light infection of *T. vaginalis* reaching **one** trophozoite which cannot be detected in traditional tests like WM (Noh *et al*., 2019). The use of traditional techniques such as WM or cultures in the diagnosis of *T. vaginalis* has not given real results about the infection rate, especially in males, so it shows a large variation in infection rates when using different diagnostic methods

such as PCR technique. In this connection, Seña *et al* (2007) reported that the IR when using urine or urethral cultures was 15.6%, while it increased to 71.7% when using the PCR technique.

RT-qPCR had the highest sensitivity (100%) and specificity (100%) for detecting *T. vaginalis* in semen samples high sensitivity of 92.4% and high specificity of 100 for detecting this parasite in urine. These findings agree with Elsherif and Youssef (2013) who found that the RT-PCR technique had a high sensitivity (99.3%) and specificity (100%). These results are in line with what was found by previous studies, that PCR test of urine samples of males was highly sensitive than the microscopic test or culture (Schwebke and Lawing 2002; Seña *et al*., 2007; Lee *et al*., 2012).

However, this result shows that all RT-qPCR amplified products of cDNA of the *T. vaginalis β-tubulin* gene in urine and semen samples were detected at the cycle threshold value (Ct value) ranged from the 19th to 21st cycles (Figure 3A). This indicates that the samples had a high abundance of nucleic acid and also indicates that the primers are specific for *T. vaginalis* and the sensitivity of the *β-tubulin* gene with RT-qPCR was high. Additionally, it shows that the peaks of PCR reactions of all positive samples of both urine and semen occurred at temperatures ranging between 75 to 78°C (Figure 3B) and the *β-tubulin* gene of each sample DNA had a single peak. These findings confirmed the opinions of Simpson *et al*. (2007) and Elsherif and Youssef (2013).

Conclusion

This study found that males in Maysan Governorate are exposed to a high rate of infection with Trichomonas vaginalis, and that the traditional method represented by microscopic examination of wet swabs is useless, so modern methods such as PCR must be used. Molecular techniques especially RT-qPCR represent the golden standard tool with high rates of sensitivity and specificity which makes it more recommended in the diagnosis of *T. vaginalis* in the fields of health, followed by RADT with moderate sensitivity and specificity, ultimately the wet mount was the less valuable method which makes it not suitable for the diagnosis of *T. vaginalis.*

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