PREDICTION OF TRICHOMONIASIS IN WOMEN COMPLAINING VAGINAL DISCHARGE BY DIFFERENT METHODS AND DETERMINE SOME IMMUNOLOGICAL MARKERS.

Zahraa Zuhair Mohammed Al- Mamoori*, Ali Atia Abid Alhisnawi1
and Jameel Jerri Yousif2

1*Department of Biology, College of Science, University of Kerbala, Iraq.
2Department of Biology, Faculty of Education for Girls, University of Kufa, Iraq.

Abstract
Trichomoniasis is the most prevalent non-viral sexually transmitted infection (STD) in the world caused by the vaginotropic extracellular protozoan parasite Trichomonas vaginalis.

The present study was conducted in Babylon Province during the period from October, 2017 to June, 2018 in AL-Hilla Teaching Hospital and AL-Zahraa Hospital, to diagnosis Trichomonas vaginalis using two methods direct microbial examination and molecular method by polymerase chain reaction using specific primer pairs of T. vaginalis B-Tubulin 9/2 and study levels of some immunoglobulins and cytokines among infected women using immunological methods. A total of (125) blood and fluid vagina samples (110) from infected women and (15) from control women were taken from women aged between (15-45) years. Two vaginal swabs (one for direct microscopical examination and the other for molecular test) and a blood sample were collected from each woman. These women suffer from acute secretions, itching, yellowish green discharge and other symptoms.

The results showed that the total frequency of infection by T. vaginalis was (20.9%) in microscope test, while in PCR technique was (13.6 %). The highest infected number and percentage of infected women was 24 (21.81%) at the age (26-35) years, while the lowest infected at the age (36-45) years was 5 (4.54%) respectively. Also the results revealed the frothy yellow to green discharge 31 (81.57%) while the malodor discharges gave 29 (23.6 8%).

The results revealed a significant increase (P<0.01) in serum concentration of proinflammatory cytokines interleukin (8 and 12) and IgG and IgM in T. vaginalis infection patients in comparison with healthy control group.

Key words: Genes; Immunoglobulin’s; Cytokines; Infected women.

Introduction
Trichomoniasis, simply, is a sexually transmitted disease typically asymptomatic in men and resulting in vaginitis with a copious, frothy discharge and itching in women, caused by Trichomonas vaginalis (Arab-Mazar and Niyyati, 2015).

Trichomonas vaginalis is an anaerobic, flagellated protozoan parasite which is the causative agent of trichomoniasis. Unlike many protozoan parasites, it possesses trophozoite form and lacks the cyst stage. The organism is most commonly isolated from vaginal secretions in women and urethral secretions in men (Kadir et al., 2014).

May have different the symptoms of infected Women with Trichomoniasis, are a frothy yellow-green vaginal discharge and vulvar irritation. While men with Trichomoniasis may have nongonococcal urethritis. The inflammation of the vaginal epithelium led to redness, swelling and leukocyte infiltration (Scott et al., 2005).

Globally, a sexually transmitted infection is considered a major public health problem. Infections with trichomoniasis estimated 143 million new infections annually (Francis et al., 2008; WHO, 2016).

There are a plethora of published studies revealed that at least 80% of T. vaginalis infections are asymptomatic. However, even asymptomatic infections...
are a public health concern. Trichomoniasis is linked to various inflammatory diseases such as prostatitis, pelvic inflammatory disease (PID) as well as to increase in the risk of Human Immunodeficiency Virus (HIV) acquisition. It is also associated with infertility in men and women as well as co-infections with different STIs (Poole and McClelland, 2013).

Diagnosis of trichomoniasis by microscopic examination is considered the most traditional method. Wet mount preparations are useful for giving clear images of fresh specimens under the microscope (Van Der Pol, 2015). The different samples can be used for the laboratory investigation of trichomoniasis. Vaginal sample (vaginal swab and vaginal secretions), urine and dried blood spot (DBS) are the sample of choices. Vaginal samples have better sensitivity than urine sample to examine the trophozoites to detect anti-trichomonal antibody by enzyme immunoassay (EIA) (Mason et al., 2005).

Enzyme immunoassay (EIA) detects exposure to trichomonal infection with >90% sensitivity in men and women (Mason et al., 2001) amplification, with increased diagnostic performance have been developed (Hobbs and Seña, 2013). These tests are validated for the detection of T. vaginalis in women and men as well as PCR is the only method currently available for rapid laboratory diagnosis (Samra et al., 2011).

Materials and Methods

Sample collection

After insertion, endocervical swab was obtained by a specialized gynecologist. The swab was inserted (1-2) cm into the endocervical canal followed by a few rotations (Siegel et al., 2007). The swab was stored in (-20°C) until using in PCR technique. By using syringe, 3 ml of venous blood was taken from each woman for the serological test. After that 3ml of the blood sample was placed in a gel tube and left standing to clot, then the tube was centrifuged at 3000 rpm for 10 minutes to collect the serum. All serum was stored in the refrigerator at -20°C until using in ELISA.

Examination of sample (wet mount examination)

Immediately, one drop from each tube was applied to a glass slide, covered with a cover slip and examined under the microscope by using the high power objective (X40) for the presence of T. vaginalis. The wet mounts were examined for at least ten minutes (Philip et al., 1987). Positive results were defined as the presence of one or more Trichomonads with characteristic motility (jerky movement) and morphology. The Trichomonads may be inactive and non-motile as in chronic or asymptomatic condition. The wet mount is also used to demonstrate the presence of clue cells in vaginal secretions; these cells were epithelial cells covered by masses of bacteria of varying morphology (Rockett et al., 2004).

DNA extraction:

• DNA extraction from vaginal swab

Before DNA extraction, swabs were taken from the freezer and left at room temperature till thawing. The vaginal cotton swab was transferred into Eppendorf tube. Extraction was performed according to the manufacturer company (FAVORGEN Genomic DNA Mini Kit Cultured Cell/USA)

PCR technique

PCR protocol was used to investigate B-tubline gene (BTUB) using primers produced by primer company-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer structure (5’ – 3’)</th>
<th>Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTUB9</td>
<td>5’ CATTGATAACGAAGCTTTCAGAT3’</td>
<td>112bp</td>
</tr>
<tr>
<td>BTUB2</td>
<td>5’ GCATGTTGGCAGCATAACCAT3’</td>
<td></td>
</tr>
</tbody>
</table>

UAS. Table 1, shows sequences of primers and PCR product sizes of BTUB and TLR4 genes.

For B-tubline gene amplification, each pcr tube has contained 12.5 µl master mix with standard buffer, 0.5 µl from each R-primer and F-primer, 5 µl of template DNA and 6.5 µl of free nuclease water (total volume :25 µl). PCR-mix tubes were closed and transferred then into the thermocycler. The amplification was performed in the PCR tubes and the procedure is as follows in table 2.

**Agarose Gel Electrophoresis**

Polymerase chain reaction products were analysed by 2% agarose gel electrophoresis (w/v) using TBE 0.5 X.

**Immuo Tests**

• Enzyme immunoassay (EIA) Detection:

• Interleukin – 8 (IL-8):

The Assay Max Human Interleukin -8 (IL-8) ELISA kit was achieved according to the manufacturing company

Table 2: Cycling parameters of genes amplification.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Steps</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTUB9/2</td>
<td>Temp. (C°)</td>
<td>94</td>
<td>94</td>
<td>60</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>30 sec.</td>
<td>30 sec.</td>
<td>45 sec.</td>
<td>45 sec.</td>
<td>5 min.</td>
</tr>
<tr>
<td></td>
<td>Cycle</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Prediction of Trichomoniasis in Women Complaining Vaginal Discharge by Different Methods

Interleukin – 12 (IL-12)

The Assay Max Human Interleukin -12 (IL-12) ELISA kit was achieved according to the manufacturing company (Elabscience/USA).

Measurement of IgG and IgM Concentration in women Infected with Trichomoniasis by Immune Diffusion Technique

The Mancini (1965) method has adopted in this test, in brief, the plate was opened and left to stand for about 5 minutes at room temperature so that any condensed water in the wells evaporated, then wells were filled with 5 µl of undiluted patient samples.

The plate was closed with the lid, the samples was left for about 20 min for diffusing into the gel, then left to stand, overturned into the envelope, at room temperature for 48 hours, then the spreading of the antibody was observed in a round shape as the diameter of the circle increased in the sample. The diameter of the ring formed in the agar plate was then measured with an ocular lens inserted from 1-20 mm and the measurements were compared with antibody concentration in the table attached with the test kit.

Statistical analysis

Data processing and the statistical analysis were performed using Statistical Package for the Social Sciences (SPSS; version 18.0). The results were given as mean ± standard deviation (Mean ± S.D). Statistical analysis for the significance of differences of the quantitative data was conducted by using ANOVA test for single factor means. Unpaired, Unequal Variances, Student’s t test used for the determination of significant differences between means of different immunoglobulin’s and cytokines used in this study and TLR4. The probability levels were indicated as follows (*p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001 and similarly for other symbols).

Results

Total percentage of Trichomonas vaginalis infection by two methods of diagnosis (Microscopic Examination and PCR).

In the present study the patient’s women were married, non-pregnant aged between 15 and 45 years. Out of (110) high vaginal swab specimens collected from women suspected of having trichomoniasis and (15) healthy women as a control group were examined by microscope and polymerase chain reaction technique, the results showed that the positive samples which was diagnosed by microscopic constitute 23 (20.9%), while those by PCR technique was 15 (13.6 %). In otherwise control after examination all individuals were negative (0% positive). These results are shown in table 3.

Table 3: Total percentage of Trichomonas vaginalis infection detected by two methods of diagnosis (Microscopic examination and PCR).

<table>
<thead>
<tr>
<th>Methods of diagnosis</th>
<th>Total samples</th>
<th>P+ (%)</th>
<th>P- (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic examination</td>
<td>110</td>
<td>23 (20.9)</td>
<td>87 (79.09)</td>
</tr>
<tr>
<td>PCR</td>
<td>110</td>
<td>15 (13.6)</td>
<td>95 (86.36)</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>0 (0)</td>
<td>15 (100)</td>
</tr>
</tbody>
</table>

Molecular techniques

• Detection of Trichomonas vaginalis using PCR diagnosis:

The T. vaginalis specific primers, PCR amplified a fragment size of 112 bp in positive test samples. No amplification was detected in the negative control samples. The results showed that the extracted DNA of swabs contain parasite DNA as shown in fig. 1, Which revealed the present of single band of amplified DNA with product size of 112 bp.

The infection percentage of T. vaginalis according to age groups

Table 4, demonstrates that the percentage of infection with trichomoniasis in women according to the age group. The highest incidence of T. vaginalis infection occurred in age group of (26-35) years with the percentage of (47.06%), followed by the age group of (15-25) years with the percentage of (32.14%) and followed by the age group of (36-45) years with the percentage of (16.13%).

Table 4: Total percentage of Trichomonas vaginalis infection by two methods of diagnosis (Microscopic Examination and PCR).

<table>
<thead>
<tr>
<th>Age group</th>
<th>T. vaginalis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-25</td>
<td>32.14</td>
</tr>
<tr>
<td>26-35</td>
<td>47.06</td>
</tr>
<tr>
<td>36-45</td>
<td>16.13</td>
</tr>
</tbody>
</table>

Fig. 1: Representative agarose gel image of the amplified PCR product from T. vaginalis DNA from collected high vaginal swabs, using red safe dye. M is the 50 bp molecular weight marker; Lane 1 is negative control; Lanes 2 - 14 are the field test samples; Lane 3, 6 and 11 were samples positive for T. vaginalis; Lane 2, 4, 5, 7, 8, 9, 10, 12, 13 and 14 were samples negative for T. vaginalis.
The infection percentage of *T. vaginalis* according to infection symptoms

• **Vaginal discharge color:**

As table 5, indicates that, the percentage of infection with trichomoniasis in women according to vaginal discharge color, the highest infection found with Yellow to green discharge which the number and percentage of infection was 31 (81.57%), followed by the women with bloody discharge 5 (13.15%), followed by the women with clear (white) discharge 2 (5.26%).

*Table 4: The infection percentage of *Trichomonas vaginalis* according to age.*

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total No. Examined women</th>
<th>No. of infected women</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-25</td>
<td>28</td>
<td>9</td>
<td>32.14</td>
</tr>
<tr>
<td>26-35*</td>
<td>51</td>
<td>24</td>
<td>47.06*</td>
</tr>
<tr>
<td>36-45</td>
<td>31</td>
<td>5</td>
<td>16.13</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>38</td>
<td>34.55</td>
</tr>
</tbody>
</table>

*The highest infection with *T.vaginalis*. *

**Table 5: The vaginal discharge color and infection with *T. vaginalis*.**

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>No.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal vaginal discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>2</td>
<td>5.26</td>
</tr>
<tr>
<td>Yellow to green</td>
<td>31*</td>
<td>81.57</td>
</tr>
<tr>
<td>Bloody</td>
<td>5</td>
<td>13.15</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>100</td>
</tr>
</tbody>
</table>

*The highest infection with *T.vaginalis*. *

• **Vaginal discharge odor:**

The results reveal that the number and ratio of the women with malodor vaginal discharge 29 (76.31%), while the women with odorless vaginal discharge had the number and percentage of 9 (23.68%), these results as shown in table 6.

*Table 6: The vaginal discharge odor and infection with *Trichomonas vaginalis*.**

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>No.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal vaginal discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Odor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malodor</td>
<td>29*</td>
<td>76.31</td>
</tr>
<tr>
<td>Odorless</td>
<td>9</td>
<td>23.68</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>100</td>
</tr>
</tbody>
</table>

*The highest infection with *T.vaginalis*. *

**Discussion**

The findings suggest that the difference between two methods is due to the results caused by different specificity and sensitivity, wet-mount microscopy, which is commonly used in routine tests, a rapid, inexpensive screening technique, but having low sensitivity shows a sensitivity of (38%) depend on the time, experience, the immediate examination of the specimen, the trophozoites loose movements after the protozoan has been removed from body temperature and may be the use of dry swabs or delayed transportation of the specimen to the laboratory these factors that contribute to low test sensitivity with wet-mount microscopy (Figueroa-Angulo et al., 2012; Nathan et al., 2015).

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 but its availability and cost effectiveness limit its use in routine diagnostic laboratories according to (Smooker et al., 2010).

The finding of the present study also revealed a highly
significant increase in the concentration of (IL-8) cytokines in serum of patient infected with *T. vaginalis* compared to the healthy control group. This result agrees with study of (Nam et al., 2012) proved that the human neutrophils and macrophages stimulated by *T. vaginalis* which produced the (IL-8) and pro-inflammatory cytokines tumor necrosis factor and IL-1β.

(Nguyen et al., 2005) revealed that the IL-1β, IL-2, IL-6 and IL-8 present in higher level which correlated with infection agent example *T. vaginalis*.

The result of (Fichorova et al., 2006) showed that LPG stimulates a significantly increased IL-8 production in the absence of cell toxicity and at low baseline levels of endogenous IL-1 and TNF-α.

These findings suggest that the production of IL-8 upon *T. vaginalis* infection may be secondary to cytopathic effects and the release of the early response proinflammatory cytokine TNF-α and IL-1β by damage epithelial cells (Al-Qadhi, 2014).

Data from the present study showed highly significant increase in the concentration of proinflammatory IFN-γ and IL-12.

In serum of patients infected with *T. vaginalis* in comparison with control group, suggesting that cytokines and chemokines provide a mechanism for initiation, amplification or containment of inflammation during disease status. This result agrees with study of (Li et al., 2018) which proved that *T. vaginalis* induced proinflammatory cytokines production in macrophages through the activation of MAPK via TLR2.

The most striking result to emerge from the data is that a significant increase in the concentration of, IgA, IgE and IgG and IgM in serum of infected with *T. vaginalis* patients compared to control group. This agrees with the experimental trichomoniasis conducted by paintlía et al., (2002) on mice infected with symptomatic and asymptomatic isolates of *T. vaginalis* alone. This increase in the concentration of IgA, IgE, IgG and IgM cooperates with increase in the B-lymphocyte which generate IgA, IgE, IgG and IgM responses (Vojdani, 2009; Finkelman et al., 1990).

Another study demonstrated that the concentration of IgG and IgM significantly increase in serum of infected with *T. vaginalis* patient in comparison with control group. This demonstrates an incitement of the humoral immune response during the infection with *T. vaginalis* (Gould et al., 2003; Kaur et al., 2008).

### Conclusions

This study showed a low prevalence of *T. vaginalis* infection in the study population.

PCR appears to be the most sensitive and specific method for detection of genital infections with *T. vaginalis*.

Also, there is a significant increase in the cytokines (IL-8 and IL-12) and immunoglobulin’s (IgG and IgM) in the serum of women infected with *T. vaginalis*.

This indicates a stimulation of the cellular and humoral immune response during the infection with *T. vaginalis*.

### References


