

## Molecular Diagnosis of the Toll-like Receptor 4 Genes and its Relationship with the Levels of some Immunoglobulin's and Cytokines among Infected Women with Trichomoniasis

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### Abstract

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*. The study was conducted in Babylon Province during the period from October 2017 to June 2018 in AL-Hilla Teaching Hospital and AL-Zahra Hospital, to diagnosis *Trichomonas vaginalis* and toll like receptor 4 using polymerase chain reaction. The relationship of TLR4 receptor with levels of some immunoglobulin and cytokines was investigated by immunological method. A total of (125) blood and fluid vagina samples were taken from women aged between (15-45) Years. The results showed that the total infection percent of *T. vaginalis* at a rate of (13.6 %) in PCR technique. And the total of TLR4 at a rate of (86.66%). The results revealed that a significant elevation ( $P < 0.01$ ) in serum concentration of pro inflammatory cytokines interleukin (4), and immunoglobulin E and A in *T. vaginalis* infection patients in compared to healthy control group. The result showed the association between IgE, IL4 and TLR4 receptor of patients.

**Keywords:** Genes; Immunoglobulin's; Cytokines; Infected women.

### Introduction

*Trichomonas vaginalis* is an anaerobic, flagellated protozoan parasite and 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 [1].

Trichomoniasis is one of the most common neglected sexually transmitted disease worldwide, which *T. vaginalis* is the causative agent. The only natural host of this parasite is humans [2].

Women with Trichomoniasis may suffer different symptoms, including a frothy yellow-green vaginal discharge and vulvar irritation. Men with Trichomoniasis may suffer nongonococcal urethritis.

The inflammation of the vaginal epithelium results in redness, swelling and leukocyte infiltration [3].

There is a large volume of published studies describing the fact 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 the risk of Human Immunodeficiency Virus (HIV) acquisition.

Further it is also associated with infertility in men and women as well as co-infections with different STIs [4]. The innate immune system is engaged to the recognition of preserved pathogen associated with molecular patterns present on the pathogen.

This recognition is facilitated by a group of receptors called toll-like receptors (TLRs), which in resulting to the synthesis and release of pro inflammatory cytokines, and consequently increase the local inflammatory response [5]. Individual TLR perceive distinct components of microorganisms, resulting in intracellular signs that lead to cell activation and cytokine production by leukocytes and other cells. The induction of a TLR4 stimulatory activity in the genital tract by *T. vaginalis* infection could result in stimulation of cells present in the genital tract or entering the genital tract.

Interestingly, in any case, epithelial cells from normal human vagina, ectocervix, and endocervix do not seem to express TLR4 and thus do not respond to lipopolysaccharide, but do express mRNA for TLR1, 2, 3, 5, and 6 [6]. Diagnosis of trichomoniasis by microscopic examination considers most traditionally method, wet mount preparations are useful for giving clear images of fresh specimens under the microscope [7].

## Materials and Methods

### Sample Collection

After insertion, endocervical swab was obtained by a specialized gynecologist after putted the patient at a lithotomic position and take swabs. The swab was inserted (1-2) cm into the endocervical canal followed by two or three rotations[12]. The swab was stored in (-20°C) until using in PCR technique.

By using syringe, 5 ml of venous blood was taken from each woman (3 ml of the serological test, 2ml of the toll-like receptor 4

genes diagnosis). After that 3ml of the blood sample was placed in a gel tube and left standing for 20 minutes at room temperature to clot, then the tube was centrifuged at 3000 rpm for 10 minutes to collect the serum. All serum was stored in the refrigerator (freezing) at -20c until using in ELISA. Then (2) ml of blood sample was placed in EDTA tube for the same people who had the infection where DNA was extracted until using in the molecular diagnosis of the toll like receptor 4 genes.

### 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 done according to the manufacturer company (FAVORGEN Genomic DNA Mini Kit Cultured Cell/USA).

#### DNA Extraction from Blood Sample

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

### Molecular Pcr Detection

Pcrprotocol has been used to investigating B-tub line gene (BTUB) and Toll like receptor gene (TLR4) using primers produced by primer company-UAS. Table (1) shows sequences of primers and PCR product sizes of BTUB andTLR4 genes.

Table 1: Sequences of primers and PCR product sizes

Primer	Primer structure (5' – 3')	Segment	Reference
BTUB9	5' CATTGATAACGAAGCTCTTTACGAT3'	112bp	[13]
BTUB2	5' GCATGTTGTGCCGGACATAACCAT3'		
TLR4-F	5'GATTAGCATACTTAGACTACTACCTCCATG3'	249bp	[14]
TLR4-R	5'GATCAACTTCTGAAAAAGCATTCCCAC3'		

For *B- tubline*& TLR4 gene amplification, each pcr tube has contained 12.5 µl master mix with standard buffer, 0.5µl from each R-primer and F-primer, 5 µl of templet DNA and 6.5µl of free nuclease water (total

volume :25 µl). PCR-mix tubes were closed and transferred then into the thermo cycler. The amplification was performed in the PCR tubes and the procedure is as follows in Table (2), (3).

**Table 2: Cycling parameters of genes amplification**

Primers	steps	Initial denaturation	Denaturation	Annealing	Extension	Final extension
BTUB9/2	Temp.(C°)	94	94	60	68	68
	Time	30 sec.	30 sec.	45 sec.	45 sec.	5 min.
	Cycle	45				

**Table 3: Cycling parameters of genes amplification10**

Primers	steps	Initial denaturation	Denaturation	Annealing	Extension	Final extension
TLR4	Temp.(C°)	94	94	60	68	68
	Time	30 sec.	30 sec.	30 sec.	1 min.	5 min.
	Cycle	30				

## Agarose Gel Electrophoresis

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

## Immuno Tests

### Enzyme immunoassay (EIA) Detection

#### Interleukin – 4 (IL-4)

The Assay Max Human Interleukin -1 Beta (IL- 1 $\beta$ ) ELISA kit was achieved according to the manufacturing company (Elabscience/USA).

#### Immunoglobulin E (IgE)

The Assay Max Human Interleukin -1 Beta (IL- 1 $\beta$ ) ELISA kit was achieved according to the manufacturing company (Calbiotech/ USA.).

### Measurement of Immunoglobulin's a Concentration in Women Infected with Trichomoniasis by Immune Diffusion Technique

The Mancini (1965) method has adopted in this test 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  $\mu$ l of undiluted patient samples.

The plate was closed with the lid, after the samples have diffused into the gel for about 20 min., then left to stand, overturned into the envelope, at room temperature for 48 hours, and then the spread of the antibody was observed in a round shape as the diameter of the circle increased in the sample and the diameter of the ring formed in the agar plate was there 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  $\pm$  standard deviation (Mean  $\pm$  S.D). Statistical analysis for the significance of differences of the quantitative data was done by using ANOVA test for single factor means.

Unpaired, Unequal Variances, Student's t test used for 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).

## Result

### Molecular Techniques

#### Detection of *T. 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 sample. The results showed that the extracted DNA of swabs contain parasite DNA as shown in Figure (1).

#### Detection of TLR4 using PCR diagnosis

The TLR4 presence was detected using specific primers pairs of TLR4 gene and the results showed the extracted DNA of blood contain TLR4 DNA as shown in Figure (2) which revealed the present of single band of amplified DNA products had 249 bp.

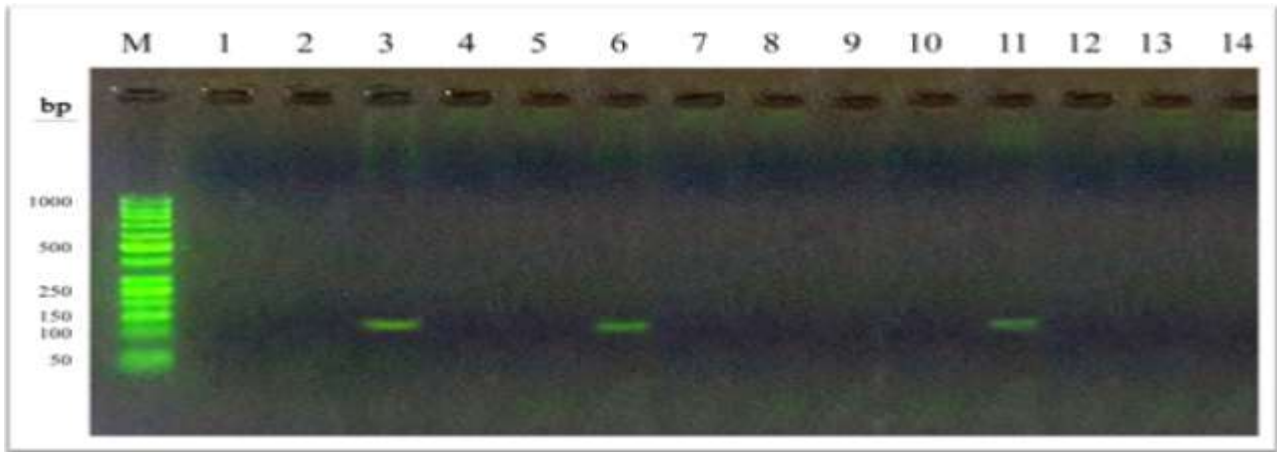


Figure 1: Representative agarose gel image of the amplified PCR product from *T. vaginalis* DNA from collected high vaginal swabs

M is the 50 bp molecular weight marker.  
 Lane 1 is a negative control.  
 Lanes 2 – 14 are the field test samples.  
 Lane 3, 6 and 11 were the positive samples for *T. vaginalis*  
 Lane 2, 4, 5, 7, 8, 9, 10, 12, 13 and 14 were the negative samples for *T. vaginalis*.

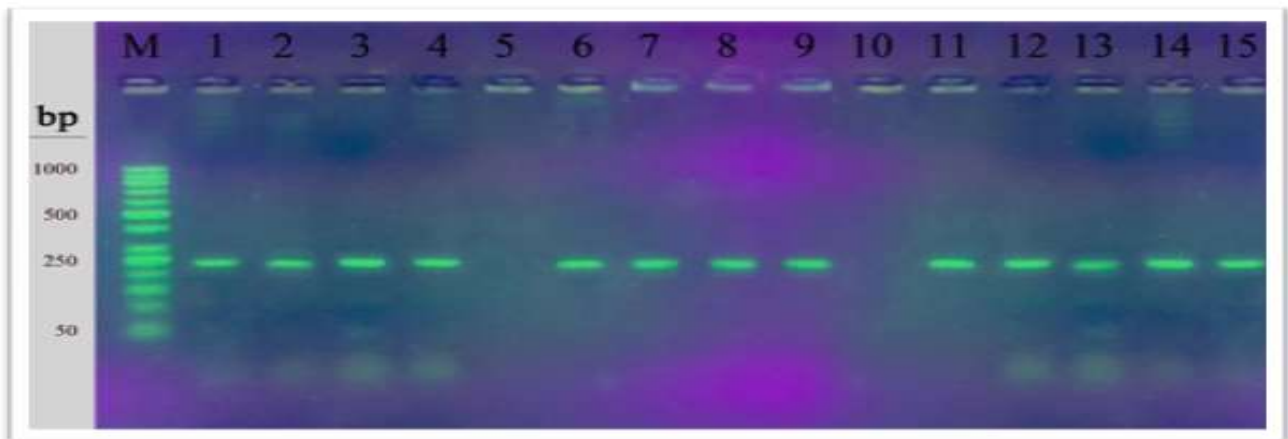


Figure 2: Representative agarose gel image of the amplified TLR4 product from DNA from collected blood

**Sero-immunological test**

**Enzyme Linked Immunosorbent Assay (ELISA)**

**Serum cytokine & immunoglobulin Levels Measurement**

The cytokine & immunoglobulin in the patients groups suffering from *T.vaginalis* infection and control group are shown in Table (4).

Table 4: cytokine & immunoglobulin Concentration (Pg / ml) in the Group of Patients Suffering from *T. vaginalis* Infections

cytokine & immunoglobulin	Healthy control (n = 15)	<i>T. vaginalis</i> patients (n = 15)
IL - 4	81.75 ± 17.3	*267.02 ± 100.61
IgA	64.02 ± 14.06	186.73 ± 73.04
IgE	118.01± 32.23	539.61 ± 142.57

**Correlation Coefficient**

With regard to aim of this study, it needed to find the relation of receptor TLR4 to some cytokines and immunoglobulin's .In the

current study there were correlations between IL-4, IgE and TLR4 receptor. Following tables show these correlations (Table 5).

Table 5: Positive correlation between TLR4 receptor and the levels of cytokines and immunoglobulins

TLR4	Factor	No.	correlation	p-value
	IL-4	15	0.591*	0.020
	IgA	15	0.084	0.765
	IgE	15	0.722**	0.002

\*. Correlation is significant at the 0.05 level (2-tailed).  
 \*\*. Correlation is significant at the 0.01 level (2-tailed).

## Discussion

Many studies nominate a role of innate and adaptive immunity in trichomoniasis. In the urogenital tract, innate immunity is accomplished by a defense physical barrier constituted by epithelial cells, mucus, and acidic pH. During infection, immune cells, antimicrobial peptides, cytokines, chemokines, and adaptive immunity evolve in the reproductive tract, and a proinflammatory response is generated to eliminate the invading extracellular pathogen *T. vaginalis*.

However, the parasite has developed complex evolutionary mechanisms to evade the host immune response through cysteine proteases, phenotypic variation, lipophosphoglycan, molecular mimicry, cytokine generation and Toll-like receptors appear to interplay with the induction of local and systemic immune responses that ultimately determine the outcome of the infection [15, 16].

As a mucosal pathogen, *T. vaginalis* must adhere to epithelial cell monolayer and once in contact with host cells, the parasite undergoes a drastic morphological shift that leads to tight association to the target cells [17]. TLRs expression plays a significant role in the innate and adaptive immune responses in epithelial cells, particularly TLR4. *T. vaginalis* infection stimulates cells through TLR4 pathway, indicating a possible immune mechanism mediated in the epithelial cells during the parasite infection [18].

"Through the recognition of pathogens or their products, TLRs can induce the production of cytokines such as IL-12 and IL-18 in APCs. These cytokines function as instructive cytokines and drive naïve T cells to differentiate into TH1 cells. Pathogens are also captured in multiple ways, including phagocytosis, endocytosis or via TLRs themselves" [19]. *In vitro* experiments have revealed that toll-like receptor 4 (TLR4) pathway is involved in the progression of immunoglobulin by induction of proinflammatory cytokines [20].

Proinflammatory cytokines have been demonstrated to be necessary for the *T. vaginalis* control and the initiation of subsequent adaptive immune response in the process of *T. vaginalis* infection [18]. Previous studies also implicated that *T.*

*vaginalis* infection induced inflammatory responses in epithelial, neutrophils, and macrophages [21,22] In general, the finding of the present study suggests that a highly significant increase in the concentration of (IL-4) cytokines in serum of patient infected with *T. vaginalis* compared to healthy control group. Increasing the (IL-4) level maybe due to increasing the monocyte or macrophages which stimulated by *T. vaginalis* infection caused vaginitis lead to stimulated host cellular and humeral immune response [23] ;this result agrees with study of young *et al.*, (2012),[24] and [25].

Al-Lihaibi *et al.*, showed a highly significant increase in the concentration of IL-4 resulted from the induction of T helper 2 (Th2) cells (CD4-positive cells) in response to the infection with *T. vaginalis*. 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 is agrees with the experimental trichomoniasis conducted by paintlia *et al.*, on mice infected with symptomatic and asymptomatic isolates of *T. vaginalis* alone.

This increase in the concentration of IgA and IgE cooperate with increase in the B-lymphocyte which generate IgA and IgE, responses [26, 27]. Another study demonstrated that the concentration of IgM, IgG and IgM significantly increase in serum of infected with *T. vaginalis* patient in compared to control group. This study concluded that there is a significant increase in the concentration of IgA and IgE due to increase in the percentage of B-lymphocyte in peripheral blood in women infected with *T. vaginalis* when compared to the control. This demonstrates an incitement of the humoral immune response during the infection with *T. vaginalis*[28,23].

The results additionally revealed that basophilia related with patients who suffering from *T. vaginalis* infection. The explanation behind this perception might be ascribed to allergy disorder which is one of symptoms of *T. vaginalis* infection these allergy causes increase IgE antibody in blood stream and these may lead to increase in the basophil because the receptor of IgE found on the surface of basophil and mast cell in human [29].

## Conclusions

PCR appears to be the most sensitive and specific method for detection of genital infections with *T. vaginalis*, Presence of TLR4 DNA in all blood samples of womern infected with *T. vaginalis*, There is a significant increase in the cytokines (IL-4) and

immunoglobulin's (IgA, IgE) 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* .Also There is a positive correlation between TLR4 receptor and the level of the cytokines (IL-4 )and immunoglobulin's ( IgE).

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