Original Article

Impact of Human Toll-Like Receptor 4 Gene Polymorphism on Infection with *Trichomonas vaginalis*

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Abstract

Background: *Trichomonas vaginalis* (*T. vaginalis*) is an anaerobic protozoan characterized by functional diagnostic flagella which are important motility tools. *Trichomonas vaginalis* triggers the innate immune system and the corresponding Toll-like receptors (TLRs). Specimens taken from infected women stimulated the TLR4-responsive cells of mice spleens to produce cytokine. **Objectives:** This genetic study aims to examine the impact of TLR4 single nucleotide polymorphism (SNP, rs4986790) on Iraqi women infected with *T. vaginalis*. **Materials and Methods:** Swabs and blood samples were isolated from 186 female patients who were admitted to the gynecology clinic in Al-Sadiq Hospital in Hilla City, Babylon province in Iraq. These samples were obtained for molecular identification of the parasite and sequencing of the TLR4 gene. **Results:** The polymerase chain reaction (PCR) analysis showed 40 females with positive PCR results (95% CI, 15.85–28.11) of the *T. vaginalis* β-tubulin gene. The frequency of A allele carriers was slightly higher (33/59) among women infected with trichomoniasis versus that in healthy controls (26/59), while the mutant G allele frequency was greater (7/11) among infected women versus controls (4/11). Moreover, the prevalence of the three genotypes, AA, AG, and GG, among infected women was 75%, 15%, and 10%, respectively ($P < 0.001$). Importantly, the homozygous GG genotype in infected women showed two times higher frequency (2/3) than controls (1/3). **Conclusion:** Although there was no statistically significant impact of TLR4 SNP (rs4986790) on susceptibility to *T. vaginalis* infection among tested women, GG genotype carriers of this SNP might have more sensitivity for trichomoniasis than other genotypes, and the AA genotype did not show protection against this infection.

Keywords: SNP, *T. vaginalis*, TLR4, trichomoniasis

Introduction

Trichomonas vaginalis (*T. vaginalis*) parasite is an anaerobic protozoan characterized by functional flagella. This parasite was first isolated from vaginal secretions by Alfred François Donné in 1836. Although the infection by this parasite is considered a mild sexually transmitted disease in humans,[1] there is an elevated incidence and prevalence rate, in addition to, an increased resistance profile to the standard treatment by metronidazole. Moreover, serious health complications were also reported which highlighted the importance of this infection.[2]

The interaction between the *T. vaginalis* parasite with the host cells was reported as a complex interaction through dependent and/or independent mechanisms. Interestingly, *T. vaginalis* does not have mitochondria, and it includes a large molecular weight genome of 176 Mbp. This genome constitutes six chromosomes, which characterizes this parasite.[3]

In humans, trichomoniasis infection produces specific antibodies in the reproductive tract against the parasite and antibodies that circulate in the serum.[4] Trichomoniasis infection, in addition, has an unclear mechanism. In detail, *T. vaginalis* triggers the innate immune system and the corresponding Toll-like receptors (TLRs). These receptors are types of transmembrane proteins, which constitute an important part of the innate response of the immune system

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against different microorganisms. In humans, 10 TLRs were reported in immune response, and combinations of these proteins were recognized in different types of human cells.[5] TLRs involve two domains: extracellular and intracellular domains. The function of the extracellular part is to recognize the ligand of the microorganism, while the intracellular part, after activation, is involved in the dimerization or association with other intracellular receptor molecules.

Trichomonas vaginalis infection produces an inflammatory response in the genitourinary tract of both genders, male and female, by TLRs stimulation. Specimens taken from cervical and vaginal regions of the infected women stimulated the TLR4-responsive cells of mice spleens to produce cytokine.[6]

Finally, the macrophage apoptosis resulting from *T. vaginalis* infection was shown to upregulate the expression of TLR4, which is recognized in the HeLa cell line after they were infected with *T. vaginalis*.[7] Consequently, the current genetic study aims to examine the impact of TLR4 single nucleotide polymorphism (SNP) (rs4986790) on Iraqi women infected with *T. vaginalis*.

Materials and Methods

Study subjects

All studied samples of *T. vaginalis* were isolated from 186 female patients. These females were admitted to the gynecology clinic in Al-Sadiq Hospital in Hilla City in Iraq between February 1st, 2022 and January 1st, 2023. The admitted females were suffering from vaginal discharge and/or itching. After taking verbal consent for participation in the study, a questionnaire was taken from the participants regarding their age, residency, history of abortion, and symptoms. The swabs collected from women's vaginas were strongly agitated with 1 mL of normal saline and then spun using a centrifuge for 10 min at 2000 × *g*. Later, the supernatant of the centrifugation product was eliminated and the pellet was frozen at −20°C for polymerase chain reaction (PCR) assay after being suspended in 1 mL of distilled water (sterile).[8,9] Additionally, 5 mL of patients' blood was collected from female participants and kept in ethylenediaminetetraacetic acid (EDTA) tubes, then these samples were frozen at −20°C which finally were utilized for DNA extraction.

DNA extraction

Wizard gDNA purification kit was used to extract 500 µL of thawed swab sample according to manufacturer instructions (Promega, Italia, Milan, Italy). For all PCR-based assays, the extracted DNAs were utilized as templates. The purity and concentration of DNA were determined by nanodrop and gel electrophoresis.

PCR amplification for diagnosis of *T. vaginalis*

Molecular identification of *T. vaginalis* strains was done by using BTUB9/2 gene-specific primers (forward 5′ CATTGATAACGAAGCTCTTTACGA T-3′, Reverse 5′ GCATGTTGTGCCGGACATAACCAT-3′) which produce—112 bp of the product. PCR assay using a final mixture volume of 25 µL was used to amplify DNA according to a previous study.[8] The 1.5% agarose gel electrophoresis was performed to analyze the PCR product and then stained with ethidium bromide to be illustrated under a Ultraviolet (UV) transilluminator. PCR mixtures used in the assay included 12.5 µL of master mix, 2.5 µL of both forward and reverse primers, 3 µL of template DNA, and 4.5 µL of nuclease-free water. PCR assay conditions involved five cycles of initialization (94°C for 5 min), denaturation (94°C for 1 min), annealing (56°C for 1 min), extension (72°C for 1 min), and final extension (72°C for 1 min).

Single nucleotide polymorphism

In this study, rs4986790 SNP (A>G) was selected in the TLR4-encoded gene to study the relationship of the trichomoniasis infection with the corresponding SNP.

Primer design

The oligonucleotide primers for the studied SNP (rs4986790) were designed in this study according to existing GenBank sequences for the studied gene reported by the "National Center for Biotechnology Information (NCBI)" (http://www.ncbi.nlm.nih.gov). These gene sequences were used to design SNP's forward and reverse primers by Primer3Plus software.[10] Oligonucleotide primers were synthesized by Macrogen Company (Korea), as shown in Table 1.

PCR amplification for single nucleotide polymorphisms loci

New uniplex-PCR conditions were used using SNP-specific primers. In this assay, uniplex-PCR was done with a total volume of 25 µL by using GoTaq® G2 Green Master Mix (Promega), through Prime 5 thermocycler (Techno, UK). PCR mixture and conditions for the rs4986790 SNP were summarized in Table 2.

PCR product sequencing

The resulting PCR products were submitted for sequencing. First, PCR products were cleaned of their content of

Table 1: rs4986790 (A>G) SNP primers with the PCR product length

SNP/Gene name	Primer sequence	Product	Study
rs4986790/TLR4	F-CCTGTCCCTGAACCCTATGA R-GCTGGTTGTCCCAAAATCAC	574 bp	New design

PCR: polymerase chain reaction

Table 2: Uniplex-PCR mixture, volumes, and conditions used for identification of rs4986790 SNP

Mixture		Conditions		
Contents of mixture	**Volume**	**Cycle type**	**Conditions**	**Cycles number**
Master mix	12.5 µL	Initialization	94°C (5 min)	1
Forward primer	1.5 µL	Denaturation	94°C (1 min)	35
Reverse primer	1.5 µL	Annealing	57°C (1 min)	
Template DNA	3 µL	Extension	72°C (1 min)	
Nuclease-free water	6.5 µL	Final extension	72°C (10 min)	1

PCR: polymerase chain reaction

Table 3: PCR-based detection of *T. vaginalis* among studied female swab samples using BTUB9/2 gene-specific primer

Outcome	Number of patients	Percentage of infection	95% Confidence interval
Positive cases	40	21.5%	15.85–28.11%
Negative cases	146	78.5%	71.89–84.17%
Total	186	100%	–

PCR: polymerase chain reaction

primer used in the amplification process by utilizing DNA fragments extraction kit (Gel/PCR) obtained from Geneaid Biotech Ltd. USA according to the manufacturer's instructions. The DNAs were purified and then sequenced by specific sequencing primers as outlined in Table 2 at Macrogen Company (Korea). Sanger sequencing technique (bi-directional) was performed by 3730xl DNA Analyzer (Applied Biosystems) from CA, USA.

Bioinformatics and statistical analysis

The primary result in data sequencing was aligned with the control sequences. GenBank sequences database reported in NCBI (http://www.ncbi.nlm.nih.gov) was used to obtain the standard sequences for alignment. In addition, Clustal W v2.0 was used to carry out multiple alignments to determine SNP, allele frequency, and genotypes[11] utilizing the Geneious Prime (V2021.1) (Biomatters, Inc., North America). The goodness-of-fit χ^2 analysis was used to test the deviation from Hardy–Weinberg equilibrium among controls. The chi-square test was further carried out to find the variations in the genotype or allele frequencies between patients and controls. The other bioinformatics as well as statistical analysis assays were carried out based on Solé *et al.*[12]

Ethical approval

The study was conducted under the ethical principles of the Declaration of Helsinki. It was carried out with patients verbally and the analytical approval was done before samples were taken. The study protocol, the subject information, and the consent form were reviewed and approved by a local ethics committee according to document number 1522 on February 16, 2022 to get this approval.

Results

Molecular identification of *T. vaginalis*

The characteristic data for detection of confirmed positive cases of *T. vaginalis* depend solely on PCR assay for detection of conserved β-tubulin gene using specific primers. As listed in Table 3, the PCR analysis performed on samples taken from 186 subjects, 40 females revealed positive PCR results (95% CI, 15.85–28.11) of the *T. vaginalis* β-tubulin gene.

The female samples of vaginal swaps were assayed by PCR molecular technique using BTUB 9/2 primer. The 40 positive cases were determined by agarose gel electrophoresis. Each set of tests involved both positive and negative controls as well as the DNA marker. The 112-bp product size was amplified in all positive samples, as demonstrated in Figure 1.

To confirm the presence of the required SNP rs4986790 (A>G), a four-color chromatogram assay was performed utilizing an automated sequencing machine to show the outcomes of the sequencing run as shown in Figure 2.

Genetic analysis

In the present study, genotyping frequency of TLR4 gene SNP (rs4986790) was detected and fitted the Hardy–Weinberg equilibrium among controls (0.004). As mentioned previously, the later model was determined using the goodness-of-fit χ^2 assay to compare the obtained frequencies of genotypes with the frequencies expected in controls. The reason for this assay is to assess an expectation that frequencies of genotypes remain constant in a population between generations, as seen in Table 4.

Comparison of the investigated TLR4 single nucleotide polymorphism in *T. vaginalis*-infected patients versus controls

The outcomes of genotyping of the amplicon of PCR products for the 40 women infected with *T. vaginalis* as well as the 30 healthy controls were sequenced using primers of TLR4, the genotype and allele frequencies of TLR4 (rs4986790) polymorphism were compared between

Figure 1: Agarose gel electrophoresis assay of uniplex-PCR outcomes using specific primers of *T. vaginalis*. Lanes M represents universal DNA ladders. Lanes 11–15 represent positive isolates (112 bp). PCR: polymerase chain reaction

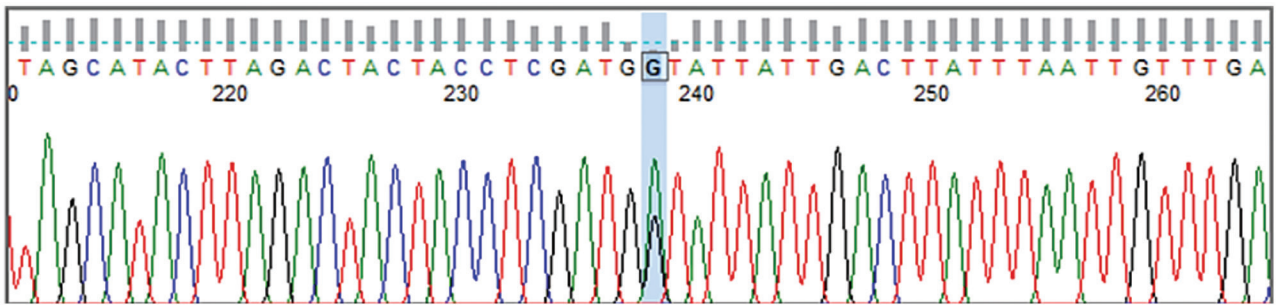

Figure 2: DNA sequencing chromatogram of TLR4 rs4986790 SNP

Table 4: An exact test for Hardy–Weinberg equilibrium (*P* value)

SNP	rs4986790
All	0.004*
Control	0.2
Patient	0.01*

*Significant difference at $P \leq 0.05$

T. vaginalis-infected patients versus controls were shown in Table 5 as well as in Figure 3. Although it is statistically nonsignificant ($P = 0.56$), the frequency of A allele carriers was higher (33/59) in women infected with trichomoniasis in comparison with that in healthy controls (26/59). On the other hand, the frequency of the G allele of the SNP was greater (7/11) in infected women in comparison with that in healthy controls (4/11). Additionally, Table 5 showed that the homozygous AA genotype frequency was slightly higher (15/27) in infected women when compared with healthy individuals (12/27). Similarly, the heterozygous AG genotype frequency was found to be slightly greater in infected women (3/5) versus controls (2/5). However, the homozygous GG genotype in infected women showed two times higher frequency (2/3) than that recorded in healthy controls (1/3), these differences were statistically not significant ($P > 0.05$).

Moreover, the prevalence of the three genotypes, AA, AG, and GG, among infected women was 75%, 15%, and 10%, respectively ($P < 0.001$), while the prevalence of the same genotypes was 80%, 13%, and 7%, respectively, in healthy controls ($P < 0.001$).

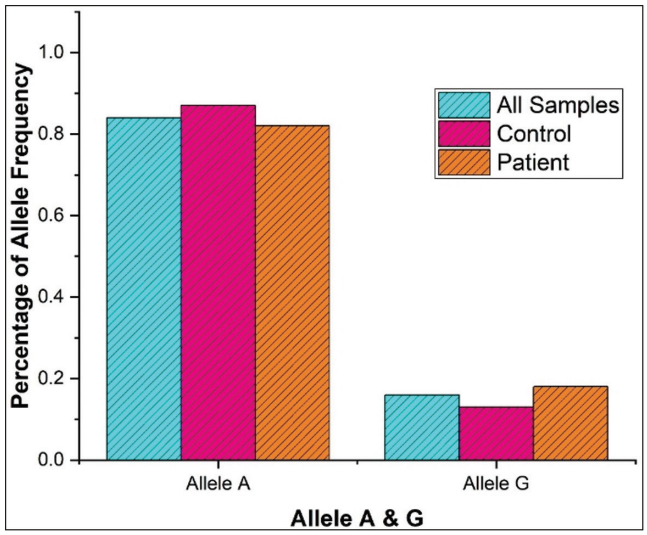

Figure 3: Distribution frequency of the TLR4 rs4986790 polymorphism between patients infected with trichomoniasis and controls

Table 6 demonstrates the frequencies of alleles of the corresponding SNP (rs4986790, A>G) in the TLR4 gene between infected patients versus controls in the current study and previous studies.

Table 5: Distribution frequency of the rs4986790 single nucleotide polymorphism in TLR4 between controls and patients infected with trichomoniasis

TLR4 SNP	Allele type	Frequency	Control population	Patients population	Significance	OR (95% CI)
rs4986790	A	59 (0.84)	26 (0.87)	33 (0.82)	$P = 0.56$	1.379 (0.364–5.22)
	G	11 (0.16)	4 (0.13)	7 (0.18)	$P = 0.56$	
	P-value	<0.001*	<0.001*	<0.001*	–	–
	Genotypes					
	A/A	27 (0.77)	12 (0.80)	15 (0.75)	$P = 0.72$	
	A/G	5 (0.14)	2 (0.13)	3 (0.15)	$P = 0.86$	1.00
	G/G	3 (0.09)	1 (0.07)	2 (0.10)	$P = 0.75$	1.20 (0.17–8.38)
	P-value	<0.001*	0.001*	<0.001*	–	−1.60 (0.13–19.84)

*Significant difference at $P \leq 0.05$

Table 6: Allele frequencies of the TLR4 single nucleotide polymorphism rs4986790 (A>G) between patients infected with trichomoniasis and controls

Population	Country	Year of publication	Patients	Controls	Reference
			(A/G) Allele frequencies	(A/G) Allele frequencies	
Caucasians	USA	2009	93.03/6.97	95.85/4.15	1000 Genomes project[13]
Asians	India	2019	86.56/13.44	92.96/7.04	[14]
Middle East	Iraq	2019	96.00/4.00	98.00/2.00	[15]
Middle East	Iraq	2023	82.00/18.00	87.00/13.00	Current study

Discussion

The genetic studies performed to evaluate the distribution frequency of the rs4986790 SNP in TLR4 between controls and patients infected with trichomoniasis demonstrated that the presence of the A allele may in part enhance the defensive mechanism against trichomoniasis for TLR4 rather than the G allele (rs4986790). Indeed, G allele carriers might be more sensitive to trichomoniasis infection than A allele. Additionally, individuals with the GG genotype, as well as the heterozygous AG genotype, could be more sensitive to infection with *T. vaginalis* than the homozygous AA genotype.

Determination of allele frequencies of TLR4 rs4986790 SNP (A>G) between patients infected with trichomoniasis and controls clearly showed that the minor allele frequency of the G allele among infected individuals is higher than that reported by previous similar studies performed locally (in Iraq) as well as in USA and India.[13,14]

In parallel, this TLR4 SNP was also reported in previous genetic studies and it demonstrated an association with sensitivity to trichomoniasis infection among individuals. rs4986790 SNP was found in two genotypes, AA and AG, in both patients and controls. The reported frequency of the AG heterozygous genotype was significantly greater in infected patients (20%) than in healthy controls (6%) (OR = 3.92, 95% CI = 1.01–15.22, $P = 0.037$). In addition, the same study found that the frequency of the mutant G allele was also significantly greater (10%) in infected patients when compared with the controls (3%).[15]

SNP in TLR4 (rs4986790) was further genetically studied in patients with other parasitic infections such as leishmaniasis, Rasouli, and his research team investigated patients diagnosed with kala-azar (visceral leishmaniasis) and healthy controls to determine the association of rs4986790 SNP with the leishmaniasis among Iranians population. However, no statistically significant association was reported of rs4986790 SNP with the corresponding infection. Furthermore, the genetic haplotypes obtained from this SNP did not differ significantly between infected patients and controls.[16]

It is still unclear how the rs4986790 SNP may modify the structure and/or activity of TLR4. However, the G allele (mutant) may alter TLR4 gene expression, signaling, or ligand binding. Of these three effects, the expression of the TLR4 gene was reported by several researchers not to be affected by these SNPs.[17,18]

In epithelial cell lines (HeLa cells) in humans, *T. vaginalis* infection elevates TLR4 expression, inducing the release of two cytokines, interleukin-8 and tumor necrosis factor-α. These two cytokines can trigger a human immune response by attracting two white blood cells, macrophages and neutrophils, at the infected sites, and enhance the T-cell activity.[7,19,20] Interestingly, ligands of TLR4 were demonstrated to control/protect cellular damage resulting from protozoan infection in the HeLa cell line.[21] Recently, it was shown that CpG oligodeoxynucleotides enhanced the survival of the *T. vaginalis* parasite of the HeLa cell line by downregulating the caspase-3 pathway and avoiding by that the apoptosis mechanism. Additionally, a previous study performed in 2013 reported no statistically

significant association between the risk of prostate cancer and *T. vaginalis* infection in the presence of TLR4 SNP (rs4986790).[22]

Another local study was performed to determine the association between TLR4 SNPs (rs4986790) with susceptibility to *T. vaginalis* infection in Iraqi women. This study concluded that no statistically significant association was imposed on susceptibility to trichomoniasis by the TLR4 SNPs (rs4986790).[23,24]

TLR4 SNP (rs4986790) was also studied in India and revealed that this corresponding SNP has increased the viral load in patients infected with HIV.[25] The mechanism behind this elevation of viral load was correlated to the role of TLR4 on the alteration of innate immune response in the mucosal surfaces, as well as changing the ability of the host to counteract the invasive pathogens rather than the interaction of TLR4 with the pathogen. Based on these findings, Ding and his research team[26] concluded that TLR4 rs4986790 SNP affects the severity of cervicitis infection among patients.

To conclude, although there was no statistically significant impact of TLR4 SNP (rs4986790) on susceptibility to *T. vaginalis* infection among tested women, GG genotype carriers of this SNP might have more sensitivity for trichomoniasis than other genotypes, and AA genotype did not show protection against this infection.

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Conflicts of interest
There are no conflicts of interest.

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