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Original Article

Immunological Role of Toll-Like Receptor Markers (TLR2 and TLR4) in Patients with *Helicobacter pylori* infection at Basrah, Iraq

Kareem, R. A[1 *], Kadhim Baqer, L[1]

1. Department of Microbiology, College of Medicine, University of Basrah, Basrah, Iraq

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Corresponding Author: lamya.bakker@uobasrah.edu.iq

Abstract

Helicobacter pylori is a spiral-shaped, flagellated, microaerophilic bacteria found in the human gastric sub-mucosa. This study aimed to investigate the association between toll-like receptor markers (TLR2 and TLR4) and the infection with *Helicobacter pylori*. The study involved 224 participants randomly divided into 2 equal groups (n=112). The patient group (n=112) was involved with several gastrointestinal symptoms. They were compared to a control group (n=112) with negative *H. pylori tests.* Patients and control were subjected to upper digestive endoscopy with gastric biopsy for the rapid urease test, rapid diagnostic test, and ELISA test for TLR2 and TLR4 detection. The recorded data showed that 36 (32.1 %) patients with *H. pylori* were in the second to the third decades of their life (25-34 years), while 22 (19.6 %) positive *H. pylori*-infected individuals were in the age range of 15-24 years, which were very close to the participants in the age range of 35-44 years. On the other hand, it is revealed that 15 (13.4%) participants were in the fourth to fifth decades of life. This rate was very similar to the groups of patients within the sixth to seventh decades of their life (13 (11.6 %)), but the lowest number of cases with *H. pylori* patients found in the age range of 55-64 years were recorded 7.1%. In conclusion, the concentration of TLR2 and TLR4 is higher in *H. pylori*-positive participants compared to the control group. This might reflect the response of innate immunity of the body to the presence of *H. pylori* infection, and thus it may be used as an ancillary tool in the detection of the patient's susceptibility to this type of infection.

Keywords: *H. pylori*, TLR 2 and TLR4, Gastrointestinal tract

1. Introduction

Helicobacter pylori is a spiral-shaped, flagellated, microaerophilic bacteria found in the human gastric sub-mucosa. Barry Marshall and Robin Warren made the initial discovery of *Helicobacter pylori* in Australia in 1982 (1). Gastrointestinal disorders like peptic ulcer, gastric cancer, and mucosa-associated lymphoid tissue lymphoma were attributed to *H. pylori* infection (2). *H. pylori* have impacted 4.4 billion individuals globally (1). The study of the *H. pylori* distribution and the clinical diagnosis among patients shows that *H. pylori* were distributed among patients with gastritis, gastric ulcer, duodenal ulcer, and gastric cancer (3). An outbreak of water pollution in 2018 in Basrah was virtually claimed to increase the rate of *H. pylori* infection in the City (4). Rates of 58% in various age groups were reported. Men were found to be more affected than women (5). Various treatment approaches were attempted. Proton-pump inhibitor (PPI) combined with amoxicillin and clarithromycin (PPI-AC) was commonly used (6).

Similarly, various diagnostic techniques were followed. They might include invasive and non-invasive approaches. Rapid urease test (RUT), culture,

and polymerase chain reaction biopsy were among the invasive tests (7). Techniques like stool antigen tests (SAT), urea breath tests (UBT), and serological investigations were non-invasive (8).

The first line of immune response is the innate pathway of which the Toll-like receptors (TLRs) are crucially identified. TLR interaction with its ligand produces a signaling cascade that initiates and maintains the secretion of antimicrobials and immune-modulating cytokines and chemokines. The innate adaptive immune system becomes activated to function suitably for the pathogen (9). TLRs play an important role in sensitizing molecular patterns. This action will bring in early recognition of foreign pathogens (10).

TLR2 plays a more significant role than TLR4 by inducing inflammatory cytokine production initiated by *H. Pylori* (11). TLR4 is essential because of its link to bacterial lipopolysaccharide (LPS), which represents the first barrier against *H. pylori*. It activates various transcription factors such as nuclear factor-*κ*B. In turn, nuclear factor-*κ*B stimulates the secretion of proinflammatory cytokines (interleukin- (IL-) 1*β*, IL-2, IL-6, IL-8, and IL-12) (12). Other studies like Loganathan, Nazeer (13) described an interaction between TLR2 and TLR4. The development of metaplasia or dysplasia and subsequent malignancy were linked to the mentioned interaction. It is well documented that the genetic polymorphism in *TLR2* is associated with a higher risk of gastric tumor. This association had variations between different geographic areas and populations.

It is well documented that TLR4 contributes to the recognition of bacterial LPS. However, TLR4 cannot recognize all types of LPSs. This weakness is attributed to the overall structural complexity of the bacterial LPSs. TLR4 is found in normal epithelial cells of the stomach lining. The release of TLR4 is distorted in cases of gastric cancer where the cancerous tissues induce LPS responsiveness to magnify the activation of NF-κB and IL-8 promoter as a result of stimulation with *H. pylori* LPS (11).

The results of a study by Li (11) demonstrated that *H. pylori* infection is related to TLR-positive cells where TLR is present on the epithelial cells and monocytes or macrophages in cases of atrophy and intestinal metaplasia of gastritis. In research, including Chinese patients with atrophic gastritis and apparent gastric cancer, it was shown that the TLR4-2081G/A polymorphism affects the likelihood of developing gastric cancer with a protective effect against *H. pylori* infection (11).

This study aimed to investigate the association between toll-like receptor markers (TLR2 and TLR4) and the infection with *Helicobacter pylori*.

2. Materials and Methods

The study involved 224 participants randomly divided into 2 equal groups involving patient and control groups (n=112) according to symptoms they had shown. The patient group was involved with several gastrointestinal symptoms. They were compared to a control group with negative *H. pylori tests.* Patients and control were subjected to upper digestive endoscopy with gastric biopsy for the rapid urease test, rapid diagnostic test, and ELISA test for TLR2 and TLR4 detection. The age range of individuals was between 15 to 74 years, with various symptoms of gastritis seeking care at the endoscopy unit at the Hospital of Gastroenterology and Hepatology in Basrah from (1 November 2021 to 15 February 2022). Patients underwent endoscopic examination.

All the patients had the main symptoms and signs like epigastric pain, bloating, vomiting and nausea. Patients who had received antibiotics, bismuth, proton pump inhibitors (PPI), or Histamine 2 (H2) receptor blockers were excluded from the study.

Specimens of gastric biopsies were obtained from the antrum and lesser curvature for each patient. Specimens were sent for diagnostic tests in order to identify *H. pylori* infection. Besides the urease test, rapid diagnostic tests that detect antibodies in the sera were also performed.

2.1. Rapid Urease Test (RUT)

The rapid urease test (RUT) was used to identify the urease enzyme in the gastric biopsy and indicates *H. pylori* infection. Figure 1 shows a sample of the RUT. After a few seconds in the positive results, the solution color changed to pink, and the negative results remained yellow.

Figure 1. A) Positive result **B)** Negative result

2.2. Rapid Diagnostic Test for Detection of *H. pylori* (RDT)

The Rapid Diagnostic Test Kit from ACON Laboratories, Inc. in the USA was used to perform a quick one-step test for the qualitative identification of IgG antibodies to *H. pylori* in human blood, as previously mentioned by Matysiak-Budnik and Megraud (14).

2.3. Immunological Study

2.3.1. Sample Preparation and TLRs Detection

From each patient (*H. pylori* positive) and control (*H. pylori* negative), 5 ml of venous blood was drawn, 2 ml (for detection of Toll-like receptors 2 and 4) was collected in the plain tube (Janetzki T24, Germany), and centrifuged for 10 minutes at 1500 rpm/min, then the separated serum was preserved at -20°C until use. To detect the concentration of TLRs 2 and 4 in patients and control ELISA specific kit (SunLong Biotech) was used. The procedure of ELISA assay was performed according to manufacturing company instructions. The concentrations of Human TLR 2 and 4 standards were known by automatically ELISA reader, as shown in figure 2.

2.4. Statistical analysis

Data were fed into SPSS, version 24, for tabulation and analysis, and the numerical data included the mean, standard deviation of the mean, median, and p-value For comparison between *H. pylori* patients and the control group.

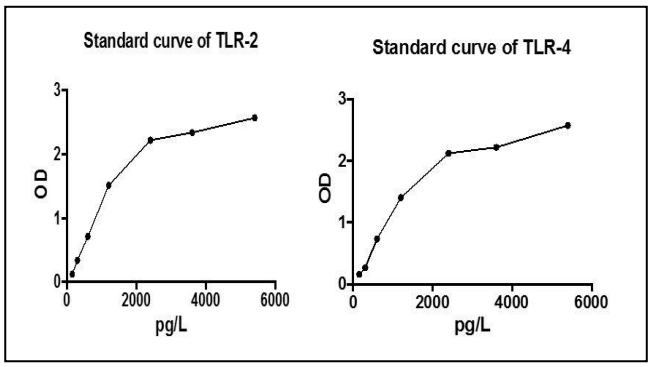

Figure 2. The stander curve of TLRs 2 and TLR4 concentration (pg/ml)

3. Results

H. pylori were detected in 112 patients via a urease test performed on biopsy specimens. In addition, they tested positive for IgG rapid tests. The majority of cases exhibited positive results as early as a few minutes. Patients with negative RUT had *H. pylori* gastritis on a clinical level and were seropositive (positive RDT). The small sample size for these individuals may cause unfavorable RUT outcomes. A favorable outcome requires at least 105 microorganisms per milliliter. It was claimed that using two biopsies boosts the test's sensitivity (15).

Table 1 showed that 36 (32.1 %) patients with *H. pylori* were in the second to the third decades of their life (25-34 years), while 22 (19.6 %) positive *H. pylori*-infected individuals were in the age range of 15-24 years, which were very close to the participants in the age range of 35-44 years. On the other hand, it is revealed that 15 (13.4%) participants were in the fourth to fifth decades of life. This rate was very similar to the groups of patients within the sixth to seventh decades of their life (13 (11.6 %)), but the lowest number of cases with *H. pylori* patients found in the age range of 55-64 years were recorded 7.1%.

Table 1. Distribution of studied group according to age group (years)

Age	Group		Total	Sig.*
	Patients	Control		
15-24	22 19.6%	24 21.4%	46 20.5%	0.998
25-34	36 32.1%	36 32.1%	72 32.1%	
35-44	18 16.1%	19 17.0%	37 16.5%	
45-54	15 13.4%	14 12.5%	29 12.9%	
55-64	8 7.1%	7 6.3%	15 6.7%	
65-74	13 11.6%	12 10.7%	25 11.2%	
Total	112 100.0%	112 100.0%	224 100.0%	

* Chi-Square Test

The recorded data showed that (Table 2) the positive *H. pylori* cases in female groups were 57 (50.9 %) versus 55 (49.1 %) for males. These differences were statistically non-significant (*P*-value=0.593).

Table 2. Distribution of studied group according to sex

Sex	Group		Total	Sig.*
	Cases	Control		
Male	55 49.1%	59 52.7%	114 50.9%	0.593
Female	57 50.9%	53 47.3%	110 49.1%	
Total	112 100.0%	112 100.0%	224 100.0%	

* Chi-Square Test

Table 3 showed that the mean level of TLRs (ng/ml) was more significant among *H. pylori* patients. The value of TLR2 in *H. pylori patients* was 2295.38±854.32 ng/ml, while the mean value for TLR4 was 2245.55±514.64 ng/ml. In the control group, the value of TLR2 was 1927.11±391.82 ng/ml, while the value of TLR4 in the control group was 1880.23±297.73 (ng/ml) (*P*=0.0001).

Table 3. The mean, median, minimum, and maximum concentration of TLRs (ng/ml)

Group		Toll Like Receptor 2	Toll Like Receptor 4
Cases	N	112	112
	Mean	2295.38	2245.55
	Median	2057.14	2057.97
	Std. Deviation	854.32	514.64
	Minimum	1756.10	1711.22
	Maximum	9246.53	5504.43
Control	N	112	112
	Mean	1927.11	1880.23
	Median	1887.54	1891.99
	Std. Deviation	391.82	297.73
	Minimum	1000.11	1021.32
	Maximum	4201.00	3457.00
Sig.*	*P*-value	0.0001	0.0001

* Mann-Whitney-U Test

The results showed that (Table 4) the level of TLR-2 (ng/ml) among males and females of *H. pylori* positive were 2170.15±461.65 and 2416.23±1100.41, respectively. In contrast, the control group's values in males and females were 1954.75±472.79 and 1896.34±276.69, respectively (*P*=0.0001).

Table 4. Comparison of TLR2 concentration (ng/ml) among studied groups concerning participants' gender

Group		Male	Female
Cases	N	55	57
	Mean±SD	2170.15±461.65	2416.23±1100.41
	Median	2000.50	2103.44
	Min.-Max.	1841-5002	1756-9247
Controls	N	59	53
	Mean±SD	1954.75±472.79	1896.34±276.69
	Median	1886.98	1895.55
	Min.-Max.	1000-4201	1002-2937
Sig.*		0.0001	0.0001

* Mann-Whitney U Test

The recorded data showed that (Table 5) the level of TLR-4 (ng/ml) among *H. pylori* positive males and females were 2197.49±408.69 and 2291.92±599.54, respectively, which was found to be higher than males and females in the control group with 1946.74±353.74 and 1806.20±197.60, respectively (*P*=0.0001).

The results showed (Table 6, Figure 3) that the TLR2 and TLR4 levels were directly proportional correlation in *H. pylori*-positive and control group R=(0.324, 0.227) respectively, statistically this correlation was highly significant with (*P*-=0.0001, 0.016) respectively.

Table 5. Comparison of TLR4 concentration (ng/ml) among studied groups concerning participants' gender

Group		Male	Female
Cases	N	55	57
	Mean±SD	2197.49±408.69	2291.92±599.54
	Median	2081.41	2044.30
	Min.-Max.	1711-3565	1838-5504
Control	N	59	53
	Mean±SD	1946.74±353.74	1806.20±197.60
	Median	1900.22	1870.31
	Min.-Max.	1045-3457	1021-2021
Sig.*		0.0001	0.0001

* Mann-Whitney U Test

Table 6. Correlation of TLR2 (ng/ml) with TLR4 (ng/ml) among *H. pylori* patient and control group

Group			Toll-like receptor 4
Cases	Toll-like receptor 2	R	0.324
		Sig.	0.0001
		N	112
Control	Toll-like receptor 2	R	0.227
		Sig.	0.016
		N	112

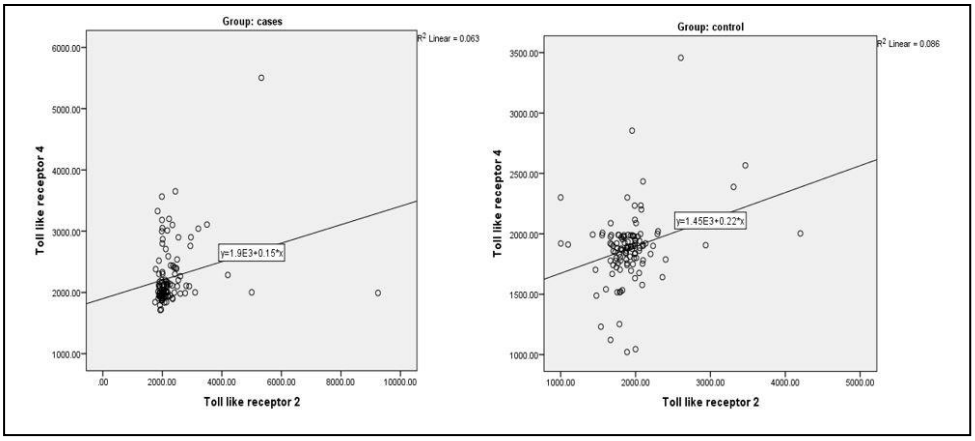

Figure 3. Correlations between TLR2 and TLR4 among *H. pylori* patients and control

4. Discussion

Half of the population in the world is infected with *Helicobacter pylori* (*H. pylori*), and this bacterium which is gram-negative found to colonize the gastric epithelium and is considered the primary cause of gastric carcinogenesis and other gastric diseases, such as chronic gastritis, gastroduodenal ulcers, and gastric mucosa-associated lymphoid tissue lymphoma (16).

This study found that the largest age group of patients with *H. pylori* was the second to the third decades (25-34) years 36 (32.1 %). These results were incompatible with research done by Bakir, Yaseen (17), who reported a high prevalence of *H. pylori* infection in the age group (> 60). The high prevalence rate in the age group (25-34) may be due to high exposure to *H. pylori* from eating outside the home or traveling to an area with a high infection rate.

In the study of gender among *H. pylori* patients and control, we documented that the cases of *H. pylori* among female groups were 57 (50.9 %) versus 55 (49.1 %) for the male group. These results agree with studies that explained that males and females are infected at the same rate (17). Also, these results were incompatible with studies by Mahmoud, Gasmi (18), which reported a significantly increased frequency of males.

Thirteen types of TLRs are known, of which types 1 to 10 are human, in contrast to 11-13, which are non-human. They are essentially expressed in immune and non-immune cells (8). This research aimed to investigate the function of Toll-like receptors in epithelial cells' response to *H. pylori* infection. TLR2 showed increased expression in *H. pylori* infected cells than those without such infection (19). Likewise, TLR4 has an increased expression in cells infected with *H. pylori* (20). As trans-membrane proteins, TLRs are essential in labeling pathogen components (21).

TLR4 is more effective in recognizing LPS of Gram-negative bacteria than TLR2. This action is through the activation of pathways that ends in an inflammatory response (22).

In the distant future, cancer may develop due to the interplay between bacterial virulence and a genetically vulnerable host, according to some scientists' theories (23).

The toll-like receptor 2 (TLR2) can reveal a variety of PAMPs on a vast set of microorganisms, like zymosan from fungi, triacyllipopeptides from bacteria, and mycobacteria, diacyl lipopeptides from mycoplasma, and peptidoglycan and lipoteichoic acid from gram-positive bacteria (8).

The current study documented that the concentrations of TLR-2 were 2295.38±854.32 (ng/ml) among *H. pylori* patients, which was higher than in control patients (1927.11±391.82). These differences may be occurred due to inflammation caused by *H. pylori* documented by Smith (9), which agrees with a study documented by AlSaimary, AlDhaheri (24), which found that the mean concentrations of TLR-2 (ng/ml) among male and female tuberculosis patients was higher than male and female of control group statistically that differences were highly significant. Hereby, TLRs are closely involved in regulating the inflammatory process throughout the innate immune response to *H. pylori.* This process represents the vital elements of activating adaptive immunity. Thus effects were made to investigate this pattern of recognizing *H pylori* and its components in various cells.

The intervention of TLR2 in NF-B activation and promoting the production of cytokines in mucosal cells infected with *H. pylori* was further validated (25).

The mean level of TLR-2 (ng/ml) among male and female positive *H. pylori* participants was more than males and females in the control group. These results show that the concentration of TLR2 in *H. pylori* female patients is slightly higher than in males, which suggests that it may occur due to the female hormone activity. In other studies, Ghatage, Anurupa (26) found that TLRs can prompt the activation of T-lymphocyte and monitor the acquired immunity, which results in a balance of the body's immune system.

Toll-like Receptor (TLR4) may play a role in developing the inflammatory process in response to *H. pylori* infection. This is true in mice and Guinea pigs (20). In the current study, it is observed that the

concentrations of TLR4 (ng/ml) among *H. pylori* positive participants was 2245.55±514.64, which was higher than the control group (1880.23-297.73). Statistically, the differences were highly significant, matching the study of Zhang (20), who demonstrated that the LPS of *H. pylori* activates TLR4 and causes the expression of the *mitogen oxidase 1* gene in Guinea pig gastric pit cells. Also, Maeda, Akanuma (27) found that the level of sTLR4 is more significant in patients with inflammation than in controls. The results of the current study also match with a study from Mustafa, Jasim (28) on Pediatric Acute Lymphoblastic Leukemia. They found that the level of TLR4 in patients is higher than in the control group, with a higher significant difference (*P*-value<0.001). Maeda, Akanuma (27), Bartova, Sommerova (29) Maeda, Akanuma (27), Bartova, Sommerova (29) Maeda, Akanuma (27), Bartova, Sommerova (29).

In this study, we found that the mean value of TLR4 (ng/ml) among males and females with positive *H. pylori* patients found to be greater than males and females in the control group; these differences were found to be highly significant which different with study from Elseweidy (30) who indicate that, despite the epithelium consider essential in the immune response against the infection with *H. pylori* but the response is independent of TLR4 at all, while it is agree with a study from AlSaimary, AlDhaheri (24) among Patients with Prostatitis whose found that the expression of all type of TLRs might be increase with the inflammatory action.

The current study's recorded data revealed a positive correlation between TLR2 and TLR4 in *H. pylori* patients. Statistically, this correlation was highly significant (*P*-value=0.0001), but there is also a positive correlation in the control group, so we cannot consider the strength of association was due to *H. pylori* infection. No previous studies on the association between TLRs (2, 4) concentration and *H. pylori* patients' age.

In conclusion, the concentration of TLR2 and TLR4 is higher in *H. pylori*-positive participants compared to the control group. This might reflect the response of innate immunity of the body to the presence of *H. pylori* infection, and thus it may be used as an ancillary tool in the detection of the patient's susceptibility to this type of infection.

Authors' Contribution

Study concept and design: R. A. K.
Acquisition of data: R. A. K.
Analysis and interpretation of data: L. K. B.
Drafting of the manuscript: L. K. B.
Critical revision of the manuscript for important intellectual content: L. K. B.
Statistical analysis: R. A. K.
Administrative, technical, and material support: R. A. K.

Ethics

The ethical approval was accepted by the ethical approval committee in the College of Medicine, the University of Basrah, and offered acceptance and approval by the research and development center, the Ministry of Health.

Conflict of Interest

The authors declare that they have no conflict of interest.

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