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ORIGINAL ARTICLE

Impact of TLR9 gene Polymorphism on Severity of *Helicobacter pylori* Infection and Its Association with Virulence genes

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ABSTRACT

Key words:
H. pylori, PCR, TLR9 gene polymorphism, virulence genes

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Background: *Helicobacter pylori colonizes stomach epithelium in the majority of people worldwide. It disturbs the local mucosa's homeostasis in the stomach and causes many pathological disorders, such as chronic gastritis, peptic ulcers and gastric cancer.* ***Objective:*** *This study aimed to determine the relation between different TLR9 gene polymorphism genotypes and the risk and severity of H. pylori -related gastric diseases and determine possible associations of these genotypes with H. pylori virulence genes.* ***Methodology:*** *136 adult dyspeptic patients who had gastric symptoms attended Gastroenterology Department at Suez Canal University Hospitals were incorporated in this study. Three stomach biopsies were obtained from each patient. The glmM gene was amplified using PCR for confirmation of H. pylori infection. Virulence genes were identified by PCR. Using PCR-RFLP, the genotypes of TLR9 gene polymorphism were analyzed.* ***Results:*** *The most prevalent genes were hsp60, vacA s1/ s2 and vacA m1/m2 detected in 93.7 % of H. pylori isolates, followed by cagA (54.2%). Frequencies of CC, TC, and TT genotypes were 41.7 %, 38.5 %, and 19.8 %, respectively in infected patients compared to 10.4 %, 54.2 %, and 35.4 %, respectively in the control group.* ***Conclusion****: This study reported a significant association between TLR9 SNP and H. pylori -related diseases and showed that the patients who had the CC genotype may have higher risk of developing severe gastritis. There may be a synergistic effect between the CC genotype and H. pylori carrying hsp60 and vacA s1/m1 genes leading to severe gastritis.*

INTRODUCTION

Over fifty percent of people worldwide have *H. pylori*, a spiral-shaped microaerophilic Gram-negative bacterium that colonizes the stomach epithelium [1]. Due to its strong correlation with gastric carcinoma (GC), the World Health Organization (WHO) has also designated it as a class I carcinogen [2].

Helicobacter pylori disturbs the local mucosa's homeostasis in the stomach and causes the synthesis of several inflammatory cytokines, which cause many pathological disorders, such as mucosa-associated lymphoid tissue lymphoma, chronic gastritis, gastric atrophy, peptic ulcers and GC. This is because of the interaction between several factors including virulence factors of these bacteria, genetic susceptibility of host, immune responses, and other environmental factors [3].

Helicobacter pylori possesses different virulence factors which include the ability to overcome the gastric acidity, high motility, and the ability of cytotoxins production[4]. The pathogenicity island cytotoxin-associated antigen (Cag) is thought to be a primary toxins formed by *H. pylori*. A bacterial type IV secretion system (T4SS) that translocate DNA and CagA into host cells is encoded by the Cag island. Intracellular CagA is accountable for the morphological changes in the cell that trigger differentiation and proliferation, ultimately leading to the formation of GC. It also triggers other signaling cascades, including proinflammatory pathways[5].

Another toxin generated by *H. pylori* and encoded by *vacA* gene is called vacuolating cytotoxin A (VacA). This is a pore-inducing toxin which causes the stomach's epithelial cells to vacuolate, which results in apoptosis[6]. Two variation regions make up the mosaic structure of *vacA* gene: the middle (m1 and m2) and signal (s1 and s2) regions, which control the toxin vacuolating activity. There is a correlation between s1/m1 and s1/m2 genotypes and high vacuolating activity. Conversely, vacuolating activity is missing in the s2/m2 genotype[7].

One of the most prevalent proteins in *H. pylori* is called heat shock protein 60 (hsp60), which functions as a molecular chaperone to protect unfolded proteins from

acid accumulation. Hsp60 plays a crucial part in the human defense mechanism by stimulating macrophages to produce interleukin 6[8].

Transmembrane proteins called toll-like receptors (TLRs) identifies PAMPs, or pathogen-associated molecular patterns, which are shared by the majority of pathogens. TLR2, TLR4, TLR5, and TLR9 are expressed by the gastric epithelium and are important components of the host's immune response against *H. pylori*[9]. Previous researches have revealed that *H. pylori*-infected gastric epithelium express more TLRs than non-infected cells. Furthermore, during the advanced phases of *H. pylori* infection, elevated TLR expressions trigger pro-oncogenes by inducing mitogen-activated protein kinases (MAPKs), which in turn induce angiogenesis and GC cell invasion via prostaglandin E2 release leading to epithelial dysplasia and adenocarcinoma[10,11]. Despite the fact that *H. pylori* infection is thought to be a substantial risk factor for stomach malignancy, only 1%–2% of patients develop GC [12].

In particular, TLR9 is an intracellular receptor that contributes to immune identification and signalling after *H. pylori* infection by identifying unmethylated CpG oligonucleotides in DNA[13]. TLR9 signalling pathway has anti-inflammatory effects as a mean of maintaining homeostasis and is crucial for protecting the gut and healing from injuries. Therefore, the changes in the TLR9 may influence the course of the *H. pylori*-associated gastric diseases [14].

The TLR9 gene's promoter region exhibits genetic diversity as the TLR9 -1486T/C (rs187084) single nucleotide polymorphism (SNP) which may cause altered expression and dysregulation of the TLR9 signalling that lead to disparity between pro- and anti-inflammatory cytokine responses with unbalanced formation of inflammatory cytokines that can causes chronic inflammation and GC [15]. However, the relation between TLR9 SNP and *H. pylori* infection's outcomes is still controversial. Therefore, we conducted this study to find the relation between different TLR9 -1486T/C polymorphism genotypes and the risk and severity of *H. pylori*‑related diseases and also determine possible associations of these genotypes with *H. pylori* virulence genes such as *cag*A, *vac*A and *hsp*60.

METHODOLOGY

Patient selection:

This case-control study was done over 10-months period from October 2022 to July 2023. 136 adult dyspeptic patients who had gastric complaints and chronic abdominal pain attending the Gastroenterology Department at Suez Canal University Hospitals (SCUHs) and received esophagogastroduodenoscopy (EGD) were selected. Individuals with malignancies, severe diseases, or histories of gastric surgery were excluded. Patients who had received bismuth compounds, proton pump inhibitors, antimicrobial medications, or H2 receptor antagonists in the four weeks prior to the endoscopic examination were also excluded.

Forty eight healthy individuals without a history of stomach complaints or anti *H. pylori* elimination therapy and confirmed negative for *H. pylori* bacteria (negative *H. pylori* antigen in stool and negative *H. pylori* antibodies) were represented as a control group. For every healthy subject, a 10-milliliter peripheral blood sample was drawn in an anticoagulant tube using strict aseptic procedures.

Every participant in our study provided a written informed consent. The Research Ethics Committee of Faculty of Medicine, Suez Canal University approved this study (Research number: 5407#).

Biopsy specimens and detection of *H. pylori* infection:

Three stomach biopsies, measuring (5 mm × 5 mm), were taken from the body and gastric antrum of each patient involved in this study by physicians during gastric endoscopy. The first biopsy was examined by a rapid urease test (RUT) to screen for *H. pylori* infection. The RUT positive remaining two biopsies one was sent to the Pathology Department for histopathological analysis and was fixed in 10% formaldehyde. Following cutting and sufficient deparaffination, formalin-fixed, paraffin embedded (FFPE) tissue was stained with Giemsa and haematoxylins and eosin (H&E) stains. The third biopsy was kept in 0.5 millilitres of normal saline and subjected to PCR confirmation. Based on histopathological results, density of *H. pylori* colonization was grouped into four categories based on the modified Sydney criteria as follows [16]:

- Absent (grade 0)
- Mild colonization (grade 1): scattered organisms covering less than one third of the surface.
- Moderate colonization (grade 2): intermediate numbers (in between).
- Severe colonization (grade 3): large clusters or a continuous layer over two thirds of surface.

DNA extraction:

From both gastric biopsy specimens and blood of healthy controls, DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Germany) based on the manufacture's rules. After that, the extracted DNA was stored at -20°C until further processing.

PCR amplification of *ureC* (*glmM*) gene for the confirmation of isolated *H. pylori* strains:

*The glm*M gene was amplified using a set of primers that are listed in **table 1.** Using the thermal cycler (Eppendorf Mastercycler gradient, Germany), PCR amplification was done at the following conditions: 3 minutes of initial denaturation at 95°C, 35 cycles of denaturation for 1 minute at 95°C, annealing for 1

minute at 55°C, and extension for 1 minute at 72°C and final extension at 72°C for seven minutes [17].

Molecular identification of *H. pylori* virulence genes *cag*A, *vac*A and *hsp*60 genes using PCR:

The following parameters were used during PCR for the *cagA* and *hsp60* genes: initial denaturation for 5 minutes at 95°C; 34 cycles of 1 minute at 94°C, 1 minute at 55°C for primer annealing, and 1 minute at 72°C for extension; the last extension step was completed for 10 minutes at 72°C [18].

PCR was done using the following cycling conditions for the virulent mosaics *vacAs1/vacAs2* and *vacAm1/vacAm2* alleles: 1 cycle at 95 °C for 1 minute, 35 cycles at 94 °C for 1 minute, 52 °C for 1 minute, 72 °C for 1 minute, and a final extension cycle at 72 °C for 6 minutes [19,20].

Genotyping of TLR9 -1486T/C, rs187084 polymorphisms in patients having *H. pylori* related gastritis and healthy controls by PCR-RFLP.

The PCR reaction was conducted using the following thermal cycling conditions: 5 minutes initial denaturation at 95°C, 35 cycles for 30 seconds at 95°C, 30 seconds at 64°C, 45 seconds at 72°C, and 5 minutes final extension at 72°C.

PCR products were examined using electrophoresis on a 2% agarose gel. Following electrophoresis, the gels were photographed under UV light transilluminator after staining with ethidium bromide**.** The primers used are showed in **table 1**.

Table 1: Primers used in the study:

Primer	Sequences (5' to 3')	Amplicon (bp)	References
*ure*C (*glm*M)	F: 5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' R: 5'-AAGCTTACTTTCTAACACTAACGC-3'	**294**	17
*cag*A	F: 5'-GATAACAGGCAAGCTTTTGAGG-3' R: 5'-CTGCAAAAGATTGTTTGGCAG-3'	**349**	18
*hsp*60	F: 5'-GCTCCAAGCATCACCAAAGACG-3' R: 5'-GCGGTTTGCCCTCTTTCATGG-3'	**603**	18
vacAs1/s2	F: 5'-ATGGAAATACAACAAACACAC-3' R: 5'-CTGCTTGAATGCGCCAAAC-3'	**s1:259** **s2:286**	19, 20
vacAm1/m2	F: 5'- CAATCTGTCCAATCAAGCGAG -3' R: 5'- GCGTCAAAATAATTCCAAGG -3'	**m1: 567** **m2: 642**	19, 20
TLR9-1486T/C, rs187084	5'-TTCATTCAGCCTTCACTCAG -3' 5'-TCAAAGCCACAGTCCACAG -3'	**558**	21

Enzymatic digestion of the PCR products:

The enzymatic reactions were done in a 20 µL reaction mixture that was incubated for an hour at 37°C. It contained 10 µL of the PCR products, 1 × reaction buffer, and 10 units of Afl II restriction enzyme (Thermo Scientific, EU, Lithuania). Following digestion, the products were separated by gel electrophoresis and visualized using ethidium bromide staining, resulting in one of three variants: an intact PCR fragment of 558 bp indicating CC genotype, two fragments of 413 bp and 145 bp indicating TT genotype, and three fragments of 558 bp, 413 bp, and 145 bp indicating TC genotype [21].

Statistical Analysis:

Data was fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Numerical and percentage representations were used for categorical data. The **Chi-square test** was applied to determine the relation between the categorical variables. As an alternative, when more than 20% of the cells had an expected count of less than 5, the **Monte Carlo correction** test was used. For continuous data, they were tested for normality by the **Kolmogorov- Smirnov** and **Shapiro-Wilk test**. The terms range (minimum and maximum), mean, standard deviation, and median were used to describe quantitative data. For quantitative variables that were normally distributed, the **student t-test** was utilized to compare two groups. The results' significance was assessed at the 5% level.

RESULTS

Among the 136 adult dyspeptic patients who had gastric complaints receiving EGD, 112 patients (82.4 %) had positive RUT. PCR was performed on all RUT positive gastric samples (n=112) for confirmation of *H. pylori* bacteria by *glm*M gene amplification. Among them, ninety-six (85.7 %) were positive for *glm*M gene and confirmed to have *H. pylori* (cases group), of them 46.9 % were males and 53.1 % were females with mean age of 49.69 ± 13 years. Among these patients, 31.3 % were smokers. 48 healthy individuals who were negative for *H. pylori* infection were included as a control group; of them 50 % were females and 50 % were males with mean age of 50.1 ± 12.8 years. Among them, 18.7% were smokers without any statistical differences between the two groups as demonstrated in **Table 2**.

Table 2: Comparison between the two studied groups according to demographic data:

	Cases (n = 96)	Control (n = 48)	Test of Sig.	p
Gender				
Male	45 (46.9 %)	24 (50 %)	χ^2= 0.125	0.723
Female	51 (53.1 %)	24 (50 %)		
Age (years)				
Mean ± SD.	49.69 ± 13	50.1 ±12.8	t= 0.164	0.870
Smoking				
Yes	30 (31.3 %)	9 (18.7 %)	χ^2= 2.532	0.112
No	66 (68.7 %)	39 (81.3 %)		

SD: **Standard deviation** χ^2: **Chi square test** **t: Student t-test**
p: p value for comparing between the two studied groups

Regarding endoscopic findings in cases (n=96), 18.8 % were presented with pan gastritis, 17.7 % with antral gastritis, 20.8 % with GERD, 17.7 % with gastric ulcer, 20.8 % with duodenal ulcer, 2.1 % with hiatus hernia and 2.1 % were normal. Histopathology revealed that 35.4 % (n=34) of patients had mild colonization with *H. pylori*, 52.1 % (n=50) had moderate colonization and 12.5 % (n=12) had severe colonization as showed in **figures 1-3**.

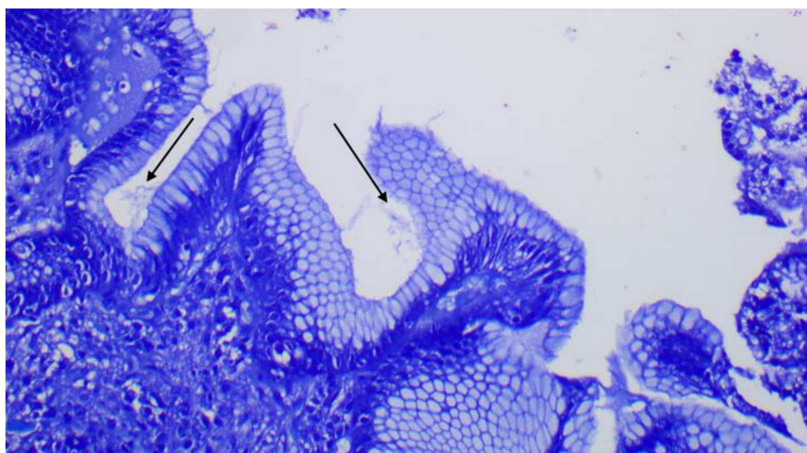

Fig. 1: Gastric antrum tissue stained with Giemsa stain and shows mild colonization with *H. pylori* in surface epithelium (black arrow) **(x400)**

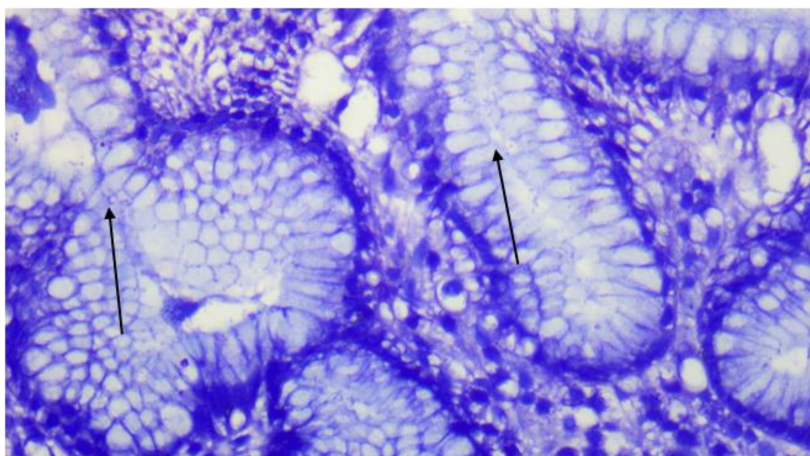

Fig. 2: Gastric antrum tissue stained with Giemsa stain and shows moderate colonization with *H. pylori* in surface epithelium and superficial glands (black arrow) **(x400)**

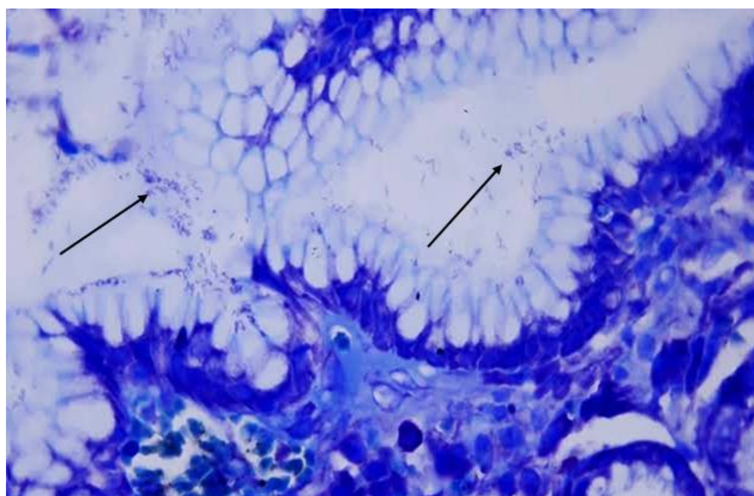

Fig. 3: Gastric antrum tissue stained with Giemsa stain and shows severe colonization with *H. pylori* in surface epithelium and superficial glands (black arrow) **(x400)**

PCR was performed on gastric biopsies from *H. pylori* infected patients for identification of *H. pylori* virulence genes. The *hsp*60, *vacA s1/ s2* and *vacA m1/m2* were the most common genes detected in 93.7 % of gastric biopsies, followed by *cagA* (54.2%). The most virulent *vacA* s1 allele was found in 82 cases (85.4 %). The middle region *vacA m*1 allele was present in 55 cases (57.3 %), while *vacA m*2 and *s*2 alleles were detected in 35 (36.4 %) and 8 (8.3 %) of the cases, respectively as illustrated in **figures 4 and 5**. Most prevalent genotype was vacA s1/m1 detected in 57.3% of patients, followed by *vacA s1/m2* (28.1%) and *vacAs2/m2* (8.3%). The *vacA s2/m1* wasn't detected in any of the patients **(Table 3)**. The combination of all genes among cases was illustrated in **table 3.**

Table 3: Frequency of *H. pylori* virulence genes among patients (n = 96)

Genes	No. (%)
cag A	
Positive	52 (54.2%)
Negative	44 (45.8%)
hsp60	
Positive	90 (93.7%)
Negative	6 (6.3 %)
vacA s1/ s2	
s1	82 (85.4%)
s2	8 (8.3%)
Negative	6 (6.3%)
vacA m1/m2	
m1	55 (57.3%)
m2	35 (36.4 %)
Negative	6 (6.3%)
Combination	
s1/m1	55 (57.3%)
s1/m2	27 (28.1%)
s2/m1	0 (0%)
s2/m2	8 (8.3%)
Combination	
+Cag A & +Hsp60 & *vacA s1/ s2* & *vacA m1/m2*	52 (54.2%)
–Cag A & +Hsp60 & *vacA s1/ s2* & *vacA m1/m2*	35 (36.5%)
–Cag A & +Hsp60 & *–vacA s1/ s2* & *–vacA m1/m2*	3 (3.1%)
–Cag A & –Hsp60 & *vacA s1/ s2* & *vacA m1/m2*	3 (3.1%)
All negative	3 (3.1%)

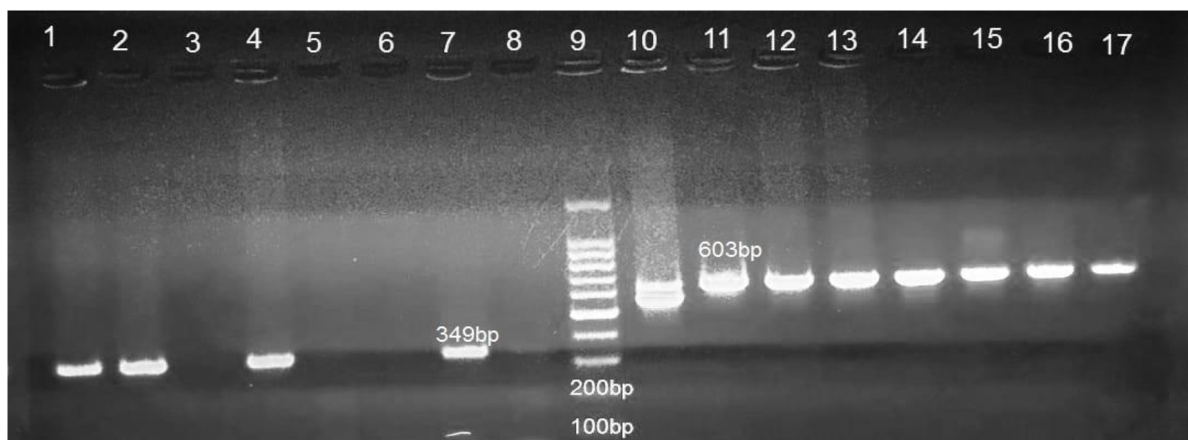

Fig. 4: PCR amplification of *cagA* (left) and *hsp60* (right) genes*:* Lane 9: 100 bp DNA ladder; lanes 1, 2, 4 and 7 positive for *cagA* gene (349 bp) and lanes 10-17 positive for *hsp60* gene (603 bp).

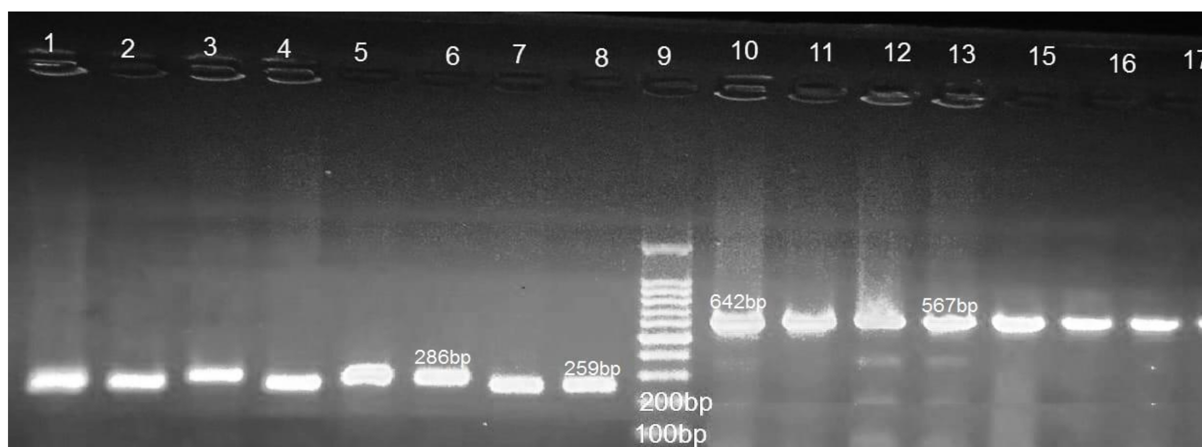

Fig. 5: PCR amplification of *vacAs1/s2* (left) and *vacAm1/m2* (right) genes*:* Lane 9: 100 bp DNA ladder; lanes 3, 5 and 6 positive for *s2* allele (286 bp), lanes 1, 2, 4, 7 and 8 positive for *s1* allele (259 bp), lanes 10-12 positive for m2 allele (642 bp) and lanes 13-17 positive for m1 allele (567 bp).

We assessed the relation between of TLR9 gene polymorphism and the risk of *H. pylori* infection. Between controls and patients infected with *H. pylori* infection, there was a statistically significant relation in TLR9 gene polymorphism distribution (P= 0.001). Frequencies of CC, TC, and TT genotypes were 41.7%, 38.5%, and 19.8%, respectively, in the patients with *H. pylori* infection, as opposed to 10.4%, 54.2%, and 35.4%, respectively, in control group. While the TC and TT genotypes were more common in control group (54.2% and 35.4%, respectively) than in case group (38.5% and 19.8%, respectively), Frequency of CC genotype was significantly higher in the cases (41.7% vs. 10.4%). The results of the logistic regression analysis indicated that the TLR9 −1486 CC genotype significantly contributed to *H. pylori* bacteria (OR=7.158; 95% CI, 2.30 –22.31; p = 0.001) **(Table 4)**.

We detected C allele frequency was higher in patients than in control (60.9 % vs. 37.5%; OR=2.600; 95 % CI, 1.57 – 4.31; p= 0.001)), whereas T allele was the most prevalent among control (62.5 %) than in cases (39.1%) as demonstrated in **table 4.** In both cases and control groups, the genotype distribution for the TLR9 -1486T/C gene polymorphism was in consistent with the Hardy-Weinberg equilibrium (p>0.05).

Table 4: Distribution of genotypes of TLR9 -1486T/C gene polymorphism in *H. pylori* infected patients and healthy controls

	Cases (n = 96) No. (%)	Control (n = 48) No. (%)	χ^2	p	OR (LL – UL 95%C.I)	p_1
Genotype						
TT	19 (19.8%)	17 (35.4%)			1.000	
TC	37 (38.5%)	26 (54.2%)	14.911*	0.001*	1.273 (0.56 – 2.90)	0.566
CC	40 (41.7%)	5 (10.4%)			7.158 (2.30 –22.31)	0.001*
^{HW}p	**0.062**	**0.281**				
Allele						
T	75 (39.1%)	60 (62.5%)	14.118*	<0.001*	1.000	
C	117 (60.9%)	36 (37.5%)			2.600 (1.57 – 4.31)	0.001*

χ^2: **Chi square test**
$^{HW}\chi^2$: Chi square for goodness of fit for Hardy-Weinberg equilibrium (If P < 0.05 - not consistent with HWE.)
p: p value for comparing between the studied groups
OR: Odds ratio CI: Confidence interval LL: Lower limit UL: Upper Limit
*: Statistically significant at p ≤ 0.05

All virulence genes *hsp*60, *cagA, vacA s1/ s2* and *vacA m1/m2* were significantly more prevalent in patients with moderate or severe colonization by *H. pylori* than in patients had mild colonization. The most common genotype among patients with mild colonization was the TT genotype representing 44.1 %, whereas the CC genotype was the most predominant among patients with moderate and severe colonization representing 54 % and 66.7 %, respectively as shown in **table 5**.

Table 5: The relation between histopathological changes with virulence genes and TLR9 -1486T/C gene polymorphism (n= 96)

	Histopathological changes			χ^2	p
	Mild (n= 34)	Moderate (n= 50)	Severe (n= 12)		
cagA					
Positive	2 (5.9%)	38 (76%)	12 (100%)	51.683*	<0.001*
Negative	32 (94.1%)	12 (24%)	0 (0%)		
hsp60					
Positive	31 (91.2%)	48 (96%)	11 (91.7%)	7.315*	^{MC}p= 0.002*
Negative	3 (8.8 %)	2 (4%)	1 (8.3 %)		
vacA s1/ s2					
s1/s2	28 (82.4%)	50 (100%)	12 (100%)	9.711*	^{MC}p= 0.004*
Negative	6 (17.6%)	0 (0%)	0 (0%)		
vacA m1/m2					
m1/m2	28 (82.4%)	50 (100%)	12 (100%)	9.711*	^{MC}p= 0.004*
Negative	6 (17.6%)	0 (0%)	0 (0%)		
Combination					
s1/m1	12(35.3%)	31(62%)	12(100%)	16.122*	<0.001*
s1/m2	8(23.5%)	19(38%)	0(0%)	7.463*	0.024*
s2/m1	0(0%)	0(0%)	0(0%)	–	–
s2/m2	8(23.5%)	0(0%)	0(0%)	13.901*	^{MC}p<0.001*
TLR9-1486T/C					
TT	15 (44.1%)	3 (6 %)	1 (8.3 %)		
TC	14 (41.2%)	20 (40 %)	3 (25%)	55.145*	^{MC}p <0.001*
CC	5 (14.7%)	27 (54 %)	8 (66.7%)		

χ^2: **Chi square test** MC: **Monte Carlo**
p: p value for comparison between the studied categories
*: Statistically significant at p ≤ 0.05

The *cagA, hsp*60, *vacA s1/ s2* and *vacA m1/m2* genes were significantly more common in patients with CC genotype than in patients with TC and TT genotypes. CC genotype of TLR9 showed a significant relation with *H. pylori* strains that carry the *cagA, hsp*60, *vacA s1/ s2* and *vacA m1/m2* virulence genes (**table 6**). GERD was the most common presentation in patients with TT and TC genotypes representing 36.8 % and 27 %, respectively, while CC genotype was most commonly presented with duodenal ulcer representing 37.5 % as shown in **table 6.**

Table 6: The relation between TLR9 -1486T/C gene polymorphism with virulence genes and endoscopic finding (n= 96)

	TLR9 -1486T/C gene polymorphism			χ^2	p
	TT (n= 19)	**TC (n= 37)**	**CC (n= 40)**		
cagA					
Positive	0 (0%)	16 (43.2%)	36 (90%)	44.921*	<0.001*
Negative	19 (100%)	21 (56.8%)	4 (10%)		
hsp60					
Positive	13 (68.4%)	37 (100%)	40 (100%)	17.197*	^{MC}p <0.001*
Negative	6 (31.6%)	0 (0%)	0 (0%)		
vacA s1/ s2					
s1/s2	14 (73.7%)	36 (97.3%)	40 (100%)	11.669*	^{MC}p= 0.001*
Negative	5 (26.3%)	1 (2.7%)	0 (0%)		
vacA m1/m2					
m1/ m2	14 (73.7%)	36 (97.3%)	40 (100%)	11.669*	^{MC}p= 0.001*
Negative	5 (26.3%)	1 (2.7%)	0 (0%)		
Combination					
s1/m1	6 (31.6%)	17 (45.9%)	32 (80%)	15.510*	<0.001*
s1/m2	3 (15.8%)	16 (43.2%)	8 (20%)	6.920*	0.031*
s2/m1	0 (0%)	0 (0%)	0 (0%)	–	–
s2/m2	5 (26.3%)	3 (8.1%)	0 (0%)	10.371*	^{MC}p=0.002*
Endoscopic finding					
Normal	2 (10.5%)	0 (0%)	0 (0%)	29.446*	^{MC}p <0.001*
Antral gastritis	5 (26.3%)	8 (21.6%)	4 (10%)		
Pangastritis	2 (10.5%)	9 (24.3%)	7 (17.5%)		
GERD	7 (36.8%)	10 (27%)	3 (7.5%)		
Gastric ulcer	2 (10.5%)	4 (10.8%)	11 (27.5%)		
Duodenal ulcer	1 (5.3%)	4 (10.8%)	15 (37.5%)		
Hiatus hernia	0 (0%)	2 (5.4%)	0 (0%)		

χ^2: **Chi square test** MC: **Monte Carlo**
p: p value for comparison between the studied categories
*: Statistically significant at $p \leq 0.05$

DISCUSSION

Helicobacter pylori is one of the primary causative agents of gastrointestinal disorders including GC. In this study, we investigated the relationships between the risk and severity of gastric diseases related to *H. pylori* and various genotypes of the TLR9.

One hundred thirty-six dyspeptic patients with gastric complaints receiving EGD were included in this study, 112 patients (82.4 %) were detected to have positive RUT. Based on PCR results, we confirmed *H. pylori* infection in 96 (85.7%) of these RUT positive patients (cases group). There were no statistically significant differences among cases and controls regarding age, sex and smoking. Similarly, multiple previous studies found no statistical significant differences among *H. pylori*-positive patients and controls regarding age, sex and smoking consumption[22,23, 24].

The prevalence of *H. pylori* in our study was 70.5 % as tested by PCR on gastric biopsies of our dyspeptic patients. This elevated prevalence was usually observed

in the developing countries especially in Egypt. A prevalence of 80 % and 90 % were detected by Khalifehgholi et al. [25] and Hunt et al. [26], respectively. However, a different study found that *H. pylori* prevalence was lower [27]. A number of factors, as low socioeconomic standards, contaminated water supplies, unhygienic living conditions, and more crowded homes, may cause a high prevalence of *H. pylori* infection in the developing countries and facilitate intra-family transmission of infection [28].

Gastritis is the earliest visible change caused by *H. pylori* infection in a stepwise process of histopathological changes, which may cause GC. Regarding the endoscopic findings in our cases, GERD and duodenal ulcer were the most prevalent findings representing 20.8 % each, followed by pan gastritis (18.8%), antral gastritis and gastric ulcer (17.7 % each) then hiatus hernia (2.1%). Histopathology revealed that 35.4 % of patients had mild colonization with *H. pylori*, 52.1 % had moderate colonization and 12.5 % had severe colonization. Similarly, a previous study conducted in Egypt found that GERD was the most frequent finding occurring in 34.7 % of patients, followed by antral gastritis (30.5 %), pan gastritis (28.4 %), diffuse erosive gastrodeuodentits (18.9 %) and peptic ulcer disease (5.3 %) [29].

More than half of *H. pylori* strains contain the cagA gene. CagA-positive *H. pylori* genotypes are related to GC and induce mucosal inflammation and production of interleukin-8 (IL-8) [30]. In our study, *cagA* gene was found in 54.2% of *H. pylori* strains. In agreement with our study, other studies reported a nearly similar *cagA* genes prevalence among *H. pylori* strains as those conducted by Akeel et al. (49.2 %) [31], Momenah et al. (52.4%) [32] and Abu Taleb et al. (57%) [33]. In contrast, a higher prevalence of *cagA* gene (81.8%) in *H. pylori* strains was detected by Kadi et al. in Saudi Arabia [34].

This study found that *hsp*60, *vacA s1/ s2* and *vacA m1/m2* were the most prevalent genes detected in 93.7 % of *H. pylori* isolates. The virulent *vacA* s1 allele was the most prevalent detected in 85.4 % of isolates. The *vacA s1/m1* genotype was the most common found in 57.3 % of cases, while the *vacA s2/m1* was not found in any of the cases.

Similar findings were detected in another earlier research. For instance, Sultan et al. in Mansoura, Egypt, discovered that 83% and 95.3%, respectively, of *H. pylori* strains had the *hsp60* and *vacA* genes. Their prevalence was 95.3% for *vacAs1/s2*, 52.8% for *vacAm1*, and 42.5% for *vacAm2* [24]. According to El-Shenawy et al. in Giza, 61.6% of the *H. pylori* strains had the *vacA* gene. The *vacAs1, vacAs2, vacAm1 and vacAm2* alleles were found in 28.3 %, 33.3 %, 18.3 % and 43.3 %, respectively of the *H. pylori* strains. They found that the most dominant genotype was *s2m2* representing 31.7 %, followed by *s1m1* (16.7 %), *s1m2* (11.7 %) and *s2m1* (1.7 %) [35]. Furthermore, Pajavand et al. in Iran discovered that, among the various *vacA* genotypes, the s2m2 and s1m2 genotypes were dominant with frequencies of 50% and 39.5%, respectively, while the s1m1 genotype [36] had a lower frequency (7.2%). These findings conflicted with our findings. Moreover, Marie in Saudi Arabia detected that the *s1/m2* genotype was the most prevalent representing 40 %, followed by the *s1/m1* genotype (28%) then *s2/m2* genotype (26%) while the *s2/m1* genotype was not detected in his study[37].

We investigated the relation between TLR9 gene polymorphism, and the risk of *H. pylori* infection and a statistically significant relation was found in the distribution of TLR9 gene polymorphism in *H. pylori*-infected patients and controls. The frequency of C allele was higher among patients than control (60.9 % vs. 37.5%; OR=2.600; 95 % CI, 1.57 – 4.31; p= 0.001)), whereas T allele was the most prevalent among control (62.5 %) than in cases (39.1%). Moreover, CC genotype frequency was higher in cases than controls (41.7 % vs.10.4 %), while the TC and TT genotypes were more prevalent among control (54.2 % and 35.4 %, respectively) than in cases (38.5 % and 19.8 %, respectively). Logistic regression analysis found a significant contribution of the *TLR9* −1486 CC genotype to *H. pylori* infection (OR=7.158; 95 % CI, 2.30 –22.31; p= 0.001).

Similar research was done in Egypt by Sultan et al. [24]who discovered that patients with GC had a higher frequency of C allele than control group (p = 0.047). Furthermore, with a p-value of 0.045, which is consistent with our results, the frequency of CC genotype in GC patients (52.8%) was higher than that of the control group (22.6%). According to our results, C allele may play a part in altering the immune system's reaction to *H. pylori* that leads to GC.

In agreement with these results, a study was done by Gao et al. found that TLR9 SNP rs187084 demonstrated a statistically significant associations with *H. pylori* infection. Minor allele homozygotes (TT) were associated with a lower risk of *H. pylori* infection when compared to their major allele homozygotes (CC), (adjusted OR =0.38, 95% CI, 0.17–0.85, p=0.02 for the rs187084 CC genotype [38]. Similarly, Xu et al. investigated the role of TLR-9 polymorphisms (C-1237T, C-1486T, and G+2848A) in susceptibility or resistance to the development of gastroduodenal ulcer in a Chinese cohort and found that TC genotype of TLR-9 C-1486T SNP was more common (48 %) in control group compared to other genotypes which agreed with our results [39].

Furthermore, Wang and his colleagues found that in China, the GC risks were significantly higher for the C heterozygote (TC) (adjusted OR=1.47, P=0.03, 95% CI = 1.04–2.10) and homozygote (CC) (adjusted OR= 1.63, P=0.04, 95% CI = 1.01–2.64) compared to the TT homozygote, indicating a dominant effect of C allele [15].

However, in Brazil, Susi et al. found that, in comparison to the CC group, the TC and TT genotypes were related to higher risk of GC (OR = 2.72, 95% CI: 1.57-4.72), P < 0.0001) [13].

On the contrary, Eed et al. detected that the distribution of TLR9 -*rs352140* genotypes showed no association with the overall *H. pylori* infection status (*P* ⍰ 0.5) and the TT genotype was found significantly higher among patients compared to control group (*P* = 0.018) [22].

Furthermore, Zhao et al. [40] found no evidence of a significant relation between *H. pylori* infection and TLR9 SNP. These disparate results could be the result of genetic variations among populations, environmental variables, and racial differences.

In this study, CC genotype of TLR9 detected a significant relation with *H. pylori* strains that carry *cagA, hsp*60, *vacAs1/ s2* and *vacA m1/m2* virulence genes. GERD was the most common presentation in patients who had TT and TC genotypes representing 36.8 % and 27 %, respectively, while CC genotype was most commonly presented with duodenal ulcer representing 37.5 %. According to Sultan et al., *H. pylori* strains that carry the *hsp60, sodB,* or *vacAm1* genes showed a significant relationship with CC genotype of TLR9 as compared to TT + TC genotypes. There was no significant correlation found between CC genotype and any of the *cagA, vacA*, *vacAm2,* or *vacAs1/s2*genes [24].

This study reported a significant relation between *H. pylori*-related diseases and TLR9 SNP rs187084 and found that patients who had CC genotype of TLR9 may have a higher risk of developing severe gastritis. Furthermore, there may be a synergistic effect between CC genotype and *H. pylori* strains that carry the *hsp60* and *vacA s1/m1* genes that leads to the occurrence of severe gastritis. However, further studies are recommended on a wider range of patients and demographics.

Ethical Approval and Consent to Participate

This study received administrative authorization from the Suez Canal University teaching Hospitals and was approved by the ethic committee of Suez Canal University (Research 5407#) and all participants provided a written informed consent before enrolment.

Conflicts of interest:

There are no conflicts of interest to report for any of the authors.

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