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The relationship between *Helicobacter pylori* disease and bacterial count in stomach

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ABSTRACT

Several studies showed significant relationships between bacterial counts and the severity and type of disease in patients. The aim of this study was to evaluate the relationship between *Helicobacter pylori* disease and bacterial count. In this study, 287 patients with dyspeptic symptoms were evaluated. Three variables including patient-reported data, clinical signs and bacterial load of gastric specimens were analyzed. Biopsy samples were collected from patients who were referred for endoscopies because of dyspeptic symptoms. Initially, the clinical status was evaluated and recorded by a questionnaire. DNA extraction was performed and *H. pylori* was analyzed for bacterial count by Real-time PCR assay and specific primers and probe. The variety range of bacteria in specimens was 10^4 to 10^{12}. The results revealed that a greater relationships existed between 10^{12} and gastric cancer (p = 0.036), also 10^4 and acid reflux (p = 0.006) and vomiting (p = 0.047). Real-time PCR assay provides a highly accurate, rapid and precise method for detection of *H. pylori* and determination of progressive disease due to this bacterium.

KEYWORDS

***Helicobacter pylori*; Gastric Disorder; Quantitative Real-Time PCR; Stomach**

1. INTRODUCTION

Helicobacter pylori is a microaerophilic, Gram-negative bacterium and is involved in the pathogenesis of peptic ulcer, gastritis, and gastric cancer [1]. Its prevalence ranges from 25% in developed countries to over 90% in developing areas [2-5]. Colonization *by H. pylori* is not a disease in itself, but as a condition that affects the relative risk of developing various clinical disorders of the upper gastrointestinal tract. It is important. Therefore, testing for the presence of *H. pylori* has no relevance to diseases but should be performed to find the cause of an underlying condition, such as peptic ulcer, or for the purpose of disease prevention, such as in subjects with a family history of gastric cancer. In these cases, a positive test result justifies treatment and a negative test result may indicate the need to search for other etiologic factors or preventive measures [3-7]. The aim of this study was to determine the *H. pylori* loads in gastric biopsy samples from patients with gastrointestinal disorders in Iran and to compare them with the severity and type of disease.

2. MATERIALS AND METHODS

287 *H. pylori* positive patients with gastrointestinal disorders in the southwest of Iran were enrolled in the study. Informed consent was taken from all patients at the beginning of endoscopy. Patient-reported symptoms and endoscopic findings by the pathologist were recorded at the time of the consultation.

For the purpose of analysis, three global variables were created: 1) patient-reported data, including age, gender and symptoms; 2) clinical signs; and 3) bacterial load as determined by PCR analysis.

Three biopsy specimens were collected from each patient (2 from antrum and 1 from corpus). All the samples

were transported to the laboratory and stored in phosphate buffer saline (PBS) until the time of assay. A rapid urease test was performed with a Gastro urease kit (Baharafshan, Iran). The DNA was isolated from each biopsy, using a DNA extraction kit (CinnaGen, Iran) according to the manufacturer's instructions and immediately used for the molecular analysis.

16S rRNA PCR analysis for the confirmation of *H. pylori* diagnosis was carried out in 25 µl reaction mixtures containing 2 µl of genomic DNA, 1.5 mM $MgCl_2$, 200 mM concentrations of dNTPs, 1 U of smar *Taq polymerase* (CinnaGen, Iran) and 0.2 mM concentrations of primers HP-1 and HP-2 under previously reported conditions [8] (**Table 1**). The 109 bp fragment was visualized after electrophoresis on a 1.5% agarose gel stained with EtBr.

Bacterial quantification was performed applying the TaqMan probe technology of real-time PCR using a Corbett 6000 (Corbett research, Australia). Real-time PCR was performed using final solution of 25 µl containing 2 µl of extracted DNA, 200 mM dNTPs, 1.5 mM $MgCl_2$, 1 U of smar *Taq polymerase* in PCR buffer 1X, 0.2 mM primers HP23S-1 and HP23S-2, and probes (**Table 1**) as previously reported under cycling conditions consisting of an initial denaturation at 95˚C for 5 min, followed by 45 cycles with denaturation at 95˚C for 30 s, annealing at 58˚C for 40 s [9,10]. All tests were performed three times on each sample. Data were analyzed with the Rotor-gene 6000 software ver1.7 (Corbett research).

Data were statistically analyzed by SPSS version 17 (SPSS Inc., Chicago, IL, USA). Statistical analysis $\chi 2$ test was used to analyze the data obtained. *P*-values less than 0.05 were considered to indicate statistical significance.

3. RESULTS

287 patients, with a median age of 51.5 (ranging from 15 to 88 years) were divided into 8 age groups from 10 to 90 years. 4.77% (137) of them were male and 52.3% (150) were female. Pain was present in 173 patients, nausea in 74, anorexia in 68, acid reflux in 55, heaviness after meals in 51, early satiety in 46, vomiting in 51 and flatulence in 70 (**Table 2**). The main endoscopic findings are depicted in **Table 3**. Chi-square analysis revealed a greater correlation between gastritis and the age group 30 - 40 ($p = 0.007$), NUD and 40 - 50 ($p = 0.001$), early satiety and 10 - 20 ($p = 0.05$), flatulence and 10-20 ($p = 0.038$), vomiting and 20 - 30 ($p = 0.001$), pain and 50 - 60 ($p = 0.003$), heaviness after a meal and 50 - 60 ($p = 0.018$) and early satiety and 60 - 70 ($p = 0.05$) (**Table 2**).

Both 16S rRNA PCR and real-time PCR assays confirmed the presence of *H. pylori* in all of these 287 biopsy samples (100%).

Mucosal bacterial quantification was performed by TaqMan real-time-PCR. The bacterial density by this technique could be evaluated for all 287 *H. pylori*-positive patients and ranged from 1.53×10^4 to 5.89×10^{12} CFU. In order to evaluate the relationship between bacterial concentration and other variables, patients were divided into 9 groups from 1×10^4 to 6×10^{12} CFU (**Table 3**). The statistical analysis revealed a greater relationship between group 10^{12} and gastric cancer ($p = 0.036$), also 10^4 and acid reflux ($p = 0.006$) and vomiting ($p = 0.047$). There was no correlation between bacterial load and age or gender.

Table 1. Sequences of oligonucleotides used in this study.

Primer/Probe	Sequence (5' → 3')	Target
HP-1 HP-2	CTGGAGAGACTAAGCCCTCC ATTACTGACGCTGATTGTGC	16*S rRNA*
HP23S-1 HP23S-2	CCACAGCGATGTGGTCTCAG CTCCATAAGAGCCAAAGCCC	23*S rRNA*
Pwt	Cy5-GGGGTCTTTCCGTCT-BHQ2	23*S rRNA*

Table 2. Distribution of symptoms and age groups.

Age group	N	Pain	Nausea	Anorexia	Acid reflux	Heaviness after meal	Early satiety	Vomiting	Flatulence
10 - 20	18	12 (6.9)	7 (9.4)	7 (10.3)	5 (9.1)	5 (9.8)	7 (15.2)*	7 (13.7)*	9 (12.9)*
20 - 30	44	27 (15.6)	14 (18.9)	13 (19.1)	12 (21.8)	5 (9.8)	5 (10.9)	16 (31.4)	8 (11.4)
30 - 40	49	25 (14.4)	16 (21.6)	11 (16.2)	11 (20.0)	6 (11.8)	6 (13.0)	6 (11.8)	11 (15.7)
40 - 50	48	31 (17.9)	6 (8.1)	12 (17.6)	5 (9.1)	10 (19.6)	3 (6.5)	10 (19.6)	9 (12.9)
50 - 60	37	27 (15.6)*	9 (12.2)	5 (7.3)	8 (14.5)	12 (23.5)*	5 (10.9)	1 (2.0)	5 (7.1)
60 - 70	44	23 (13.3)	10 (13.5)	8 (11.8)	9 (16.4)	6 (11.8)	12 (26.1)*	4 (7.8)	15 (21.4)
70 - 80	24	14 (8.1)	5 (6.7)	6 (8.8)	5 (9.1)	-	3 (6.5)	3 (5.9)	5 (7.1)
80 - 90	23	14 (8.1)	7 (9.4)	6 (8.8)	-	7 (13.7)	5 (10.9)	4 (7.8)	8 (11.4)
Total	287	173	74	68	55	51	46	51	70

*Statistically significant.

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Table 3. Interview data and endoscopic findings according to bacterial count of *H. pylori*.

	Variables	10^4 (n = 12)	10^5 (n = 18)	10^6 (n = 121)	10^7 (n = 100)	10^8 (n = 22)	10^9 (n = 4)	10^{10} (n = 4)	10^{11} (n = 4)	10^{12} (n = 4)	Total
Symptoms	Pain	5 (1.7)	16 (5.6)	73 (25.4)	56 (19.5)	10 (3.5)	4 (1.22)	4 (1.4)	4 (1.22)	1 (0.3)	173 (60.3)
	Nausea	5 (1.7)	2 (0.7)	35 (12.2)	23 (8)	5 (1.7)	-	-	2 (0.6)	2 (0.7)	74 (25.8)
	Anorexia	5 (1.7)	5 (1.7)	31 (10.8)	25 (8.7)	0 (0)	2 (0.6)	-	-	-	68 (23.7)
	Acid reflux	7 (2.4)	2 (0.7)	21 (7.3)	16 (5.6)	7 (2.4)	-	-	-	2 (0.7)	55 (19.2)
	Heaviness after meal	2 (0.7)	5 (1.7)	16 (5.6)	21 (7.3)	5 (1.7)	-	2 (0.7)	-	-	51 (17.8)
	Early satiety	3 (1)	2 (0.7)	23 (8)	14 (4.9)	2 (0.7)	-	2 (0.7)	-	-	46 (16)
	Vomiting	5 (1.7)	4 (1.4)	21 (7.3)	17 (5.9)	2 (0.7)	-	-	-	2 (0.7)	51 (17.8)
	Flatulence	5 (1.7)	7 (2.4)	33 (11.5)	19 (6.6)	2 (0.7)	-	2 (0.7)	-	2 (0.7)	70 (24.4)
Diseases	Peptic Ulcer	1 (0.3)	-	12 (4.2)	7 (2.4)	4 (1.4)	2 (0.6)	-	-	-	26 (9.1)
	Gastric Cancer	-	-	-	-	-	-	1 (0.3)	-	4 (1.4)	5 (1.7)
	Gastric Erosion	2 (0.7)	4 (1.4)	35 (12.2)	31 (10.8)	9 (3.1)	2 (0.6)	-	3 (1)	-	86 (30)
	Nodularity	4 (1.4)	7 (2.4)	23 (8)	19 (6.6)	5 (1.83)	-	-	-	-	58 (20.2)
	Gastritis	5 (1.7)	5 (1.7)	26 (9.1)	23 (8)	2 (0.7)	-	2 (0.7)	-	-	63 (22)
	Duodenitis	-	2 (0.7)	2 (0.7)	2 (0.7)	-	-	-	-	-	6 (2.1)
	Atrophy	-	-	-	4 (1.4)	1 (0.3)	-	-	-	-	5 (1.7)
	Duodenit Ulcer	-	-	23 (8)	14 (4.9)	1 (0.3)	-	-	-	-	38 (13.2)

4. DISCUSSION

In the present study, we have developed a real-time PCR assay based on the amplification of a fragment of the 23S rRNA gene with the TaqMan technology aimed at detecting *H. pylori* directly from gastric biopsy specimens, quantifying the bacterial density, and evaluating the relationship between bacterial load and disease severity. This assay provides formal statistical proof that the concentration of *H. pylori* in the gastric system is extremely high in gastric cancer patients.

The quantitative sensitivity of our assay was 400 bacteria per reaction tube, *i.e.*, 800 copies of the 23S rRNA gene. As few as 40 bacteria (80 copies of the gene) could be detected but not accurately quantified. Therefore, our real-time PCR can accurately quantify the gastric mucosal density of *H. pylori*. Other techniques can be employed for quantification; however, each technique has its weaknesses. A bacterial culture is only semiquantitative and time-consuming. Histology is also semiquantitative, but its accuracy is relatively low because of great interobserver variation. The urea breath test has been shown to be uncorrelated to culture-determined bacterial density [11].

Several studies demonstrate that there are significant correlations between bacterial counts and the severity and type of disease in patients infected with *S. enterica* serovar typhi [12], *Orientia tsutsugamushi* [13], and *M. Tuberculosis* [14], but so far not enough results regarding *H. pylori* have been reported.

H. pylori infection results in a sequence of events, ultimately resulting in the development of some gastrointestinal disorders. The initial acute gastritis is followed by active chronic gastritis, which lasts for life if the infection is not treated. Nevertheless, *H. pylori*-positive subjects are mostly unaware of this inflammation due to the lack of clinical symptoms. The ongoing presence of *H. pylori* and the resulting cellular damage initiates the histological cascade including gastric atrophy, intestinal metaplasia, dyspepsia and finally gastric cancer [4,6]. Our study was consistent with these findings: the bacterial load was 10^5 to 10^6 in biopsies from patients with chronic active gastritis, 10^7 to 10^8 in gastric atrophy and 10^{12} in gastric cancer. In this study, we found a correlation between a bacterial count of 10^4 and some symptoms including vomiting and acid reflux. The simultaneous presence of these symptoms may indicate infection by *H.*

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pylori and the possible development of the *H. pylori* disease cascade.

In conclusion, we show that disease severity and clinical symptoms are dependent on the bacterial load. *H. pylori*-positive patients are often described as being "completely well". When the *H. pylori* load in gastric mucosa increases, the first clinical symptoms occur, including pain, anorexia, heaviness after meal, early satiety, nausea and flatulence. Finally, when a bacterial load of 10^4 is reached, vomiting and acid reflux are induced. After bacterial colonization, when the bacterial count reaches 10^5, the first grade of disease is observed. This induces an inflammatory response which leads to some lesions and consequently to acute gastritis, which is followed by active chronic gastritis when bacterial counts reach 10^6. This lasts for life if the infection is not treated. The ongoing inflammation and cellular damage due to more colonization with *H. pylori* lead to the second grade of disease severity with bacterial counts of 10^7 that induce gastric atrophy. When the bacterial density reaches high levels, gastric cancer appears. These data were collected from our findings and those of other investigators. We believe more researches in other regions of the world are needed to confirm the relationship between the number of bacteria and different stages of gastric disease.

Lower frequency of sever diseases such as gastric cancer and atrophy in our samples is a limitation of this study. Therefore, it is suggested that this evaluation should conduct on large samples with a higher number of sever disorders and in different areas.

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