

Prevalence of *Helicobacter pylori* in Formaldehyde Fixed Paraffin Embedded Gastric Tissues of Gastric Cancer Patients by Scorpion Real-Time PCR Assay

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Background: Gastric cancer is the second leading cause of cancer-related deaths worldwide and it seems that environmental and lifestyle factors and infection with *Helicobacter pylori* (*H. pylori*) have a major role in the etiology of gastric cancer.

Objectives: The aim of this study was to investigate the presence of *H. pylori* DNA in archival gastric tissues of patients with gastric cancer disease by rapid, sensitive and specific technique of Scorpion real-time PCR.

Patients and Methods: This retrospective cross-sectional study was performed during the year 2009, on 285 paraffin embedded gastric specimens of patients, who were pathologically proved to have gastric cancer and were admitted to Bou-Ali, Shahid Rajaie and Dehkhoda Hospitals and Bahar and Farzam Private Laboratory of Qazvin city, Iran.

Results: The results of the Scorpion real-time PCR showed that *H. pylori* DNA was present in 8.42% of the total specimens. Modified McMullen's staining of paraffin embedded sections were positive in ten patients. There was no significant relationship between the presence of *H. pylori*, sex, age and place of residence.

Conclusions: Although the existence of *H. pylori* in gastric tissue samples of patients with gastric cancer is controversial however, our results showed that in our studied specimens a significant number of patients with gastric cancer had *H. pylori* colonization.

Keywords: Scorpion; Real-Time PCR; *Helicobacter pylori*; Cancer

1. Background

Gastric cancer is the second leading cause of cancer-related deaths worldwide. The International Agency for Research on Cancer reported that in Asia malignancy of the stomach is the most common cancer and nearly two-thirds of this disease occurs in developing countries (1). Although the incidence and mortality of distal gastric cancers has decreased in the Western world, the incidence of proximal tumors is increasing in the male population of these regions. In contrast, in Asian countries distal tumors have increased while proximal tumors have decreased (2). This geographic variation accompanied by differences in lifestyle, time trends and migratory effects associated with the incidence of gastric cancer suggest that environmental and lifestyle factors have a major role in the etiology of gastric cancer (3, 4). Gastric cancer is an outcome of a complex interaction between host and environmental factors and *Helicobacter pylori* (*H. pylori*) infection. Scientific evidence suggests the importance of host factors in gastric cancer pathogenesis (5). *Helicobacter pylori* are gram-negative, microaerophilic and spiral-shaped bacteria. *Helicobacter pylori* infection affects 50% of the population, worldwide (6). This infection

initiates an inflammatory response, which is influenced by genetic polymorphisms, and in turn can either accentuate or attenuate the host's response to inflammation and affect the interaction of *H. pylori*, host and environmental factors. *Helicobacter pylori* infection is an established and important causal factor for non-cardia gastric adenocarcinoma. In the process of gastric carcinogenesis, bacterial virulence factors that have been implicated include the cytotoxin-associated gene A antigen (Cag A), vacuolating cytotoxin (VacA), and outer membrane proteins (OMP) (7). The *cagE* genotype has been associated with gastric cancer in some studies, but contrary results have also been published (8, 9). Regardless of differences in researches opinion about the prevalence of *H. pylori*, an association between this infection and gastric cancer has been claimed by several reports in the literature (10-17).

2. Objectives

We planned this study to determine the overall prevalence of *H. pylori* infection in patients with gastric carcinoma in our setup.

3. Patients and Methods

This retrospective cross-sectional study was performed on 345 paraffin embedded gastric specimens of patients who were pathologically proved to have gastric cancer and were admitted to Bou-Ali Shahid Rajaie and Dehkoda Hospitals and Bahar and Farzam Private Laboratory of Qazvin city, Iran, during 2009.

3.1. Clinical Samples

Initially, the paraffin embedded gastric tissues were sectioned into 50 μm slices followed by DNA extraction using a DNA preparation protocol obtained from the Section of Cancer Genomics, Genetics Branch, NCI, Institute of Health Modified McMullen's Staining of Gastric Biopsy. The paraffin embedded sections were dewaxed, dehydrated and covered by carbol fuchsin for two minutes. The sections were rinsed and stained with malachite green for two minutes. Then the slides were rinsed in tap water and air dried. With this procedure gull shaped *H. pylori* were stained in magenta against light green gastric tissue.

3.2. Scorpion Real-Time

Scorpion real-time PCR was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystem, USA). The tails of the Scorpion primers were designed as previously described by Burucoa (18). All primers and probes used were synthesized by the Metabion Company (Germany) (Table 1). The total volume of the real-time PCR was 20 μL containing 5 μL of DNA from a clinical sample or bacterial isolate, 0.1 μM of oligonucleotide primer 23SF2 and 0.08 μM of 23SScWT, and the rest of the volume consisted of distilled water. The cycling conditions were adjusted in a way suitable to be used by the ABI Prism 7500 Sequence Detection System (Applied Biosystem, USA) with an initial denaturation at 95°C for 45 seconds, 50 cycles at 95°C for 15 seconds, 55°C for 34 seconds, and 72°C for 20 seconds. Acquisition of a signal was achieved at 57°C during each cycle. A negative control for the Scorpion real-time PCR was obtained by observation of no amplification signal except internal amplification by adding DDW instead of DNA to the prepared real-time PCR master mix. The positive control for the Scorpion PCR was extracted DNA from *H. pylori* ATCC 26695. Positive extraction control was performed for each biopsy specimen in a separate tube in a final volume of 25 μL with Premix Ex Taq (TaKaRa, Shiga, Japan), 5 μL of extracted DNA from biopsy specimen, 0.25 μM of BGLO1 and BGLO2 primers, and 0.5 x Sybergreen 1 (Sigma Aldrich); on the basis of the results of this test, 285 of the collected biopsies were entered in our study. The cycling program consisted of one cycle at 95°C for 10 seconds and 40 cycles at 95°C for 5 seconds, 55°C for 34 seconds, and 72°C for 10 seconds. The Ethical Committee of Qazvin University of Medical Sciences approved our study. Data were analyzed using the SPSS 11.5 software with 95% confidence intervals (95% CI).

Table 1. Scorpion Primers and Probe Sequences

Oligonucleotide	Sequence
23SScWT	5'-FAM AAGGTAGGTGAAAATCCTCCTAC-CBHQ1HEGGGACACGGGGTCTTT-3'
23SF2	5'-TGCGAACTGTGTGTTCACTAGC-3'
BGLO1	5'-ACACAACGTGTGTTCCTAGC-3'
BGLO2	5'-CAACTTCATCCAACGTTCCACC-3'

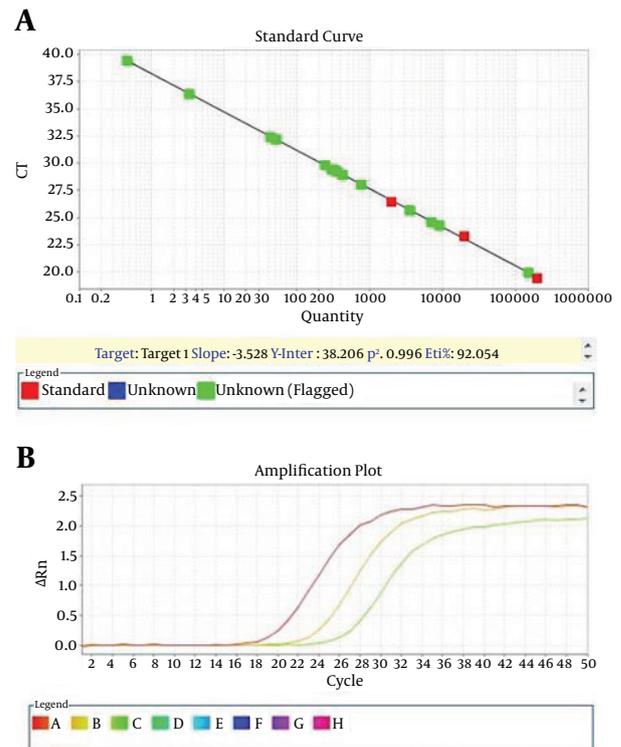
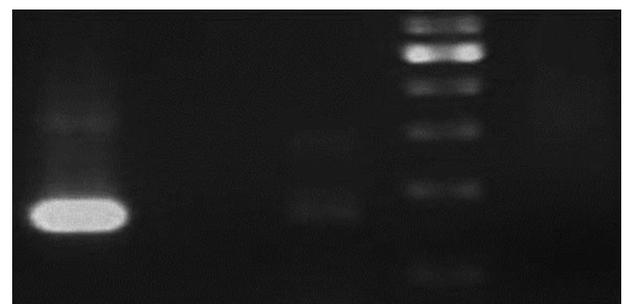


Figure 1. Standard Curve Used to Calculate the Concentration of *H. pylori* DNA in Unknown samples

Figure 2. The PCR Product was Electrophoresed by Agarose Gel Electrophoresis and Stained by Ethidium Bromide



Lane 1; *H. pylori* ATCC26695 was the positive control; lane 2, negative control; lane 3, patients' positive sample; lane 4, 100 bp DNA ladder.

4. Results

In this survey the prevalence of *H. pylori* infection in gastric samples of patients with gastric cancer was determined using the data obtained from the following experiments:

Detection of *H. pylori* by Scorpion real-time PCR: initially, Scorpion real-time PCR was performed on *H. pylori* ATCC 26695. From the total of 285 samples, 24 biopsies were found to be positive for *H. pylori* by the Scorpion real-time PCR whereas 261 samples had negative results. Among samples with positive results for the Scorpion real-time PCR test, 10 samples also had positive results for the modified McMullen's staining.

Scorpion real-time PCR: Scorpion real-time PCR on DNA extracts of *H. pylori* ATCC 26695 as the control strain produced the expected signals. The standard curve used to calculate the concentration of *H. pylori* DNA in unknown samples is presented in Figure 1A. The linear regression coefficient was 0.996 and the efficiency of PCR was 92.054%. Figure 1B shows the fluorescent curves of the standard serial dilutions from 1×10^4 to 1×10^2 copies per microliter. Electrophoresis pattern of the Scorpion real-time PCR products on agarose gel is shown in Figure 2. A 140 base pair (bp) fragment in the peptidyl transferase gene of 23S rRNA showed successful amplification. Amongst all patients (285 cases) with gastric cancer entered in the study, 24 cases (8.24%) were found to have *H. pylori* DNA in their gastric biopsy specimens by the Scorpion real-time PCR method. Our study showed that there is no significant association between sex, age, marital status, history of gastric involvement, gastric disease, and the presence of bacterium. Also no relationship was established between sex, family income, and accommodation variables and the colonization of *H. pylori* in gastric cancer tissues (data not shown).

5. Discussion

Helicobacter pylori infect humans during childhood and until now it has infected one half of the world's population. It is the only bacterium, which is listed amongst microbial agents involved in human cancer diseases. Our findings showed that from the total of 285 formaldehyde fixed paraffin embedded samples of pathologically proved gastric cancer patients entered in our study, 24 patients (8.42) had *H. pylori* DNA. The results of published researches in the medical literature related to the involvement of *H. pylori* in gastric cancer are controversial and confirmation of this involvement and determination of the level of this involvement in different societies are important issues. As *H. pylori* is a fastidious bacteria, microbiological methods of culture and identification of these bacteria are difficult and require a long duration of time, thus adequate sensitivity and specificity of modern molecular methods in diagnosis of these bacteria make them attractive methods for studying the prevalence of *H. pylori* in different clinical specimens (19). Khanna et al.

in a study on 50 proved cases of gastric cancer found that the prevalence rate of *H. pylori* infection in their patients was lower than in the control population, suggesting that *H. pylori* may not be responsible for gastric carcinogenesis in this population (16). In another study by Rudi and colleagues on the sera of 111 Caucasian patients with histologically confirmed gastric cancer, their data did not provide evidence that the contribution of *H. pylori* infection to the carcinogenesis of gastric cancer is of major significance, at least in a population with low gastric cancer rates and high socioeconomic status (17). On the other hand, Babus and colleagues analyzed 12 prospective case-control studies and concluded that between 65% and 80% of non-cardia gastric cancers were attributable to *H. pylori* infection (12, 13). Uemura et al. studied 1526 Japanese patients, of whom 1246 had *H. pylori* infection and 280 were not infected. Over a mean follow-up period of 7.8 years, gastric cancer developed in 2.9% of patients with *H. pylori* infection while none of the uninfected patients developed gastric cancer, resulting in a relative risk of 34.5 (14). Martinez and colleagues in a case-control study including 46 gastric cancer cases and 99 controls with non-atrophic gastritis from a high-risk zone for gastric cancer showed that there is a positive association between *Helicobacter pylori* CagA positive strains with non-cardia gastric cancer etiology (15). Although we studied a relatively large population and used the sensitive and specific method of Scorpion real time PCR, our results are in consistent with the latter studies indicating a possible role for *H. pylori* in gastric cancer.

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