Prevalence of cytotoxin-associated genes of *Helicobacter pylori* among Iranian GERD patients

Aref Shavalipour¹, Habib Malekpour², Hossein Dabiri¹,², Hossein Kazemian¹, Homayon Zojaji⁴, Mahboube Bahroudi⁴

¹ Department of Medical Microbiology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
² Basic and Molecular Epidemiology of Gastrointestinal Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases Shahid Beheshti University Medical Sciences, Tehran, Iran
³ Department of Medical Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
⁴ Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

**Aim:** Since the impact of *H. pylori* and its virulence is not clear in GERD, this study aimed to evaluate the prevalence of *cag A* and *cag E* genes of *H. pylori* among Iranian GERD patients.

**Background:** Gastroesophageal reflux disease (GERD) is defined as a condition of reflux the stomach juice by low pH causes tissue damage. *Helicobacter pylori* may or may not influence the GERD; however, it is unclear.

**Methods:** This study was a case-control study performed on patients with GERD who underwent upper gastrointestinal endoscopy at Taleghani Hospital of Tehran, Iran. Prevalence of *H. pylori* and presence of the *cag A* and *cag E* genes in GERD and control group was investigated.

**Results:** *H. pylori* was detected in 54% and 62% of GERD and control groups respectively. Prevalence of *cag A* gene among GERD patients was 44.4% whereas among the control group it was 87%. Prevalence of the *cag E* among GERD patients and control group was 44.4% and 64% respectively. Coexistence of *cag A* and *cag E* in GERD patients was 25.7% and in the control patients it was 54.8%.

**Conclusion:** We did not find correlation between *H. pylori* existence in GERD patients in comparison to the control group. Similar to other Asian studies, the presence of the *cag A* in control group was more than GERD patients significantly. The co-existence of *cag A* and *cag E* was also more in control group significantly.

**Keywords:** *Cag A*, *Cag E*, *Helicobacter pylori*, GERD, Iran.

(Please cite as: Shavalipour A, Malekpour H, Dabiri H, Kazemian H, Zojaji H, Bahroudi B. Prevalence of Cytotoxin-associated genes of *Helicobacter pylori* among Iranian GERD patients. Gastroenterol Hepatol Bed Bench 2017;10(3):178-183).

Introduction

Gastro esophageal reflux disease (GERD) is defined as a condition in which the reflux of the stomach juice by low pH causes tissue damage. GERD’s prevalence in Western countries is about 20% and it is 2.5% – 6.7% in Asian population; however, the prevalence is increasing in Asian populations (1). According to previous studies, *Helicobacter pylori* may or may not influence the GERD; however, it is unclear (2).

*H. pylori* is a Gram negative micro-aerophilic spiral shaped bacteria that colonize the gastric lumen of humans and other primates (3). Infection with *H. pylori* occurs worldwide, but the geographical prevalence varies greatly from 90% in developing countries to 20–50% in developed countries (4).

It can be the major cause of peptic ulcer and gastritis and is known to have a relation with some infectious and non-infectious diseases, such as parasitic infection,
malignancy, autoimmune thyroid disease, and GERD (4-6).

Cytotoxin-associated gene products (Cag A and Cag E) are virulence factors of H.pylori which contribute to disease progress (7). Previous studies suspected that Cag A of H.pylori plays a role in the pattern of infections and diseases such as GERD (8, 9). Polymerase chain reaction (PCR) technique that has sensitivity and specificity for the diagnosis of H.pylori is also pathologic methods has these characteristics so these are the gold standard for diagnosis of H.pylori (37,38,39).

Many epidemiological studies demonstrated a negative association between H. pylori infection and GERD. Some of the virulence factors, such as Cag A, may affect the diverse prevalence of GERD. A high incidence of Cag A-positive isolates has been reported in Asian population. The variety of H. pylori infection in Eastern and Asian countries among GERD patients is attributed to Cag A gene. So far, many studies have evaluated the effects of H. pylori eradication among GERD patients, but it is inconclusive. Eradication of Cag A positive H. pylori leads to recovery of acid secretion capacity and corpus gastritis which might be the causes of the higher incidence of GERD in Asian population (2). Of the cag pathogenicity island genes, the cagE gene (cytotoxin associated gene E), is also related to an increased production of IL-8 in the gastric epithelial cells (41). Thereby the cagE is an important marker of pathogenicity alone or combined with cagA (40).Since the impact of H. pylori and its virulence in GERD development is not yet well clear, so we aimed to evaluate the prevalence of H. pylori as well as its major Cag pathogenesis island markers including Cag A and Cag E gens in GERD patients compare to control group referring to Taleghani Hospital, Tehran, Iran.

Methods

Participants

The current case-control study was performed on patients who had undergone endoscopy at Taleghani Hospital of Tehran, Iran during one year (2014).

According to various studies in Iran, the average prevalence of GERD patients was 27% (10). The number of patients with GERD symptoms was calculated with the following formula: \( N = \frac{z^2 P (1-P)}{d^2} \).

(Prevalence (P) = 0.27; z = 1.96; d = 0.05)

Therefore, during the study, 303 cases were investigated. All patients were examined by a gastroenterologist. Also questionnaires was loaded for each patient prior to biopsy. Based on gastroenterologist's diagnosis (upper gastrointestinal endoscopy and physician examination), only 50 patients were diagnosed with GERD. Of other patients without Gastro esophageal reflux disease, 50 patients were considered as control group equal to GERD group. The presence of H. pylori infection in the subjects was determined by histological examination and detection of the ure C gene by polymerase chain reaction (PCR).

Biopsy

After a fasting period, upper endoscopy was performed with a standard forward-viewing endoscope. After inspection of the entire gastric mucosa, multiple biopsies were taken from the stomach. During endoscopy samples taken from the gastric antrum were placed in the the sterile microtube and was transferred to the laboratory. The specimens were preserved at -20 C for next steps.

Histological examination

The biopsy samples of the gastric antrum destined for histology were fixed in formalin and stained with Hematoxylin-Eosin (H&E) and Giemsa. (42)

| Table 1. Primers sequences used in the current study |
|-----------------------------|-----------------------------|
| **Gene** | **Primer designation** | **Sequence** | **Reference** |
| Ure C | Ure CR1 | GCTTACTTTTCTAACCACACGGGG | 37 |
|       | Ure CF1 | GATAAGCTTTTAGGGGTGTTAGGGG |   |
| Cag A | CagA F1 | AACAGGACAGTGAGCAGCAGC | 37 |
|       | CagA R1 | TATTAATGCGTGTGCTGCTGCTG |   |
| Cag E | CagE F1 | GCCGCAGATAAACAACCTCTTACA | 36 |
|       | CagE R1 | CAAGCCCATTAGGATCATTGCTG |   |
DNA extraction and PCR performing

DNA of biopsy samples was extracted by DNA extraction kit (DNeasy 96 Blood & Tissue Kit, Qiagen, USA). To confirm the presence of *H. pylori* among samples, PCR reaction was performed for *Ure C* gene. After identification of *H. pylori* positive samples, PCR was carried out for *cag A* and *cag E* genes as described previously (11) (Table 1 the primer were mentioned) (36).

**Statistical analysis**

All data were analyzed using SPSS 22. For compression of the presence of *H. pylori* among GERD patients and control group, *Cag A* and *Cag E* between the two groups, Fisher’s exact test and Chi-Square were used. A *P* value of <0.05 was considered as significant.

**Results**

Out of 50 GERD patients, 42% were male (n=21) and 58% female (n=29) with the mean age of 45.78 ±22 years. In the control group, 54% were male and 46% female with mean age of patients with the mean age of 41.27±18 years. The *ure C* PCR results in the GERD group showed that in 54% (n=27) of patient samples, *H. Pylori* DNA was detected whereas 62% (n=31) of the control group showed positive results for *ure C* gene (figure 1). And about *ure C* in control group and GERD group we cannot found statistical significance was seen in presence of *H. pylori* between the GERD and control groups. Prevalence of the *cag A* gene among GERD patients was 44.4% (n=12) whereas among the control group was 87% (n=27) (figure 2). Prevalence of the *cag E* among GERD patient was 44.4% (n=12) whereas among the control patients it was 64% (n=20) (figure 3). Coexistence of the *cag A* and *cag E* as in GERD patients was 25.7% (n=7) and in the control patients it was 54.8% (n=17) (Table 2).

**Discussion**

*H. pylori* infection plays a major role in the pathogenesis of peptic ulcer disease, chronic gastritis, and development of gastric cancer. However, its role in reflux diseases such as GERD is not clear (12-14).

![Figure 1](image1.png)

**Figure 1.** PCR results for the *ureC* gene in *H. pylori* isolates.
Line1: positive sample for the *ure C*, Line2: Size marker (50bp), Line3: Positive control for *ure C*, Line4: Negative control for the *ure*.

![Figure 2](image2.png)

**Figure 2.** PCR results for the *cagA* gene in *H. pylori* isolates.
Line1: Negative sample for the *cagA* gene, Line2: positive sample for the *cagA* gene, Line3: Size marker (50bp), Line 4: Positive control for the *cagA* gene, Line5: Negative control for the *cagA* gene.

Table 2. Prevalence of the *cag A* and *cag E* genes among GERD and control patients.

| Patients | number | *H. pylori* | Virulence genes (%) |
|----------|--------|-------------|---------------------|
|          |        | negative    | positive            |
|          |        | 23          | 27                  |
| GERD     | 50     | 12(44.4)    | 12(44.4)            |
| Control  | 50     | 19          | 31                  |
|          |        | 27(87)      | 20(64)              |
|          |        | Co-presence | Co-presence         |
|          |        | 7(25.7)     | 17(54.8)            |
| P value  | -      | 0.54        | 0.0007              |
|          |        | 0.18        | 0.03                |
GERD reduces the patients’ quality of life and imparts a significant economic burden on the healthcare system (15–17). Bacterial virulence and host inflammatory responses are important in determining the patterns of acid secretion and gastritis (18).

The incidence of *H. pylori* infection in patients with GERD varies widely from 30 to 90% (19). Geographical location of the studies due to the difference in the prevalence of *H. pylori* in the world is the reason of this heterogeneity (20). These epidemiological data do not support a causative role of *H. pylori* for reflux disease, but they suggest a negative association (21). Consistent to Johnson LF et al. study, we did not find a significant association between *H. pylori* prevalence among the GERD patients and control group. Other researchers have even found a lower incidence of *H. pylori* infection in patients with GERD and have suggested a "protective" role of *H. pylori* against the GERD (22, 23). Their findings are inconsistent with our results.

In Asian populations, in contrast to Far East and European populations, patients with gastric ulcer get complicated by corpus-predominant gastritis, which is characterized by atrophy of acid-secreting glands due to gastric acid hypo-secretion (24, 25). Gastric acid hypo-secretion prevents the development of GERD. According to previous Asian population-based studies, the prevalence of GERD is reported to have a lower prevalence (26-29); this confirms the theory. Also, *H. pylori* and GERD have been found to be negatively associated and strongly dependent on cytotoxin-associated gene product Cag A positive strains (8). Recently, a study reported that *H. pylori* Cag A positive may potentially protect against development of GERD (30-31). According to previous studies, most Cag A positive strains in Asian countries were East Asian Cag A positive strains which can protect people against GERD (18). On the other hand, it has been reported that eradication of Cag A positive *H. pylori* strains is a risk factor for newly developed GERD (32-33). A meta-analysis study demonstrated that eradication of Cag A positive *H. pylori* was related to a higher risk of developing GERD in Asian studies (34). Also another study demonstrated a strong negative association between Barrett’s esophagus or erosive esophagitis and *H. pylori*, particularly in Cag A positive strains (35). our finding in Iranian subjects, similar to many studies as well as Xie T et al. report, showed that the cag A positive *H. pylori* strains were less common among GERD patients in comparison to the control group (*P* = 0.0001) but we did not find any association between Cag E in the GERD patients and control group. The co-existence of Cag A and Cag E in the control group was more than GERD patients significantly (*P* = 0.034). Our finding was supportive for the protective role of *H. pylori* with the cagA/cagE positive genotype against GERD development. Anita P et al showed prevalence of cagE in GERD patients is more from the genes studied, but no association was detected between cagE genotypes and clinical outcome (43). The paradox is that our study is probably due to the geographical distance between the two studies.

In Conclusion, we evaluated the prevalence of the Cag A and Cag E genes of *H. pylori* among GERD patients. We didn’t find any correlation between *H. pylori* frequency in GERD patients in comparison with the control group. However in accordance with several Asian studies, *H. pylori* strains from GERD patients were less positive for cagA gene as well as co-existence of cagA/cagE compared with the control group indicating probably protective role of these factor against GERD. However, more studies are needed to
confirming this correlation and finding the possible mechanisms accurately.

Acknowledgment
This work was supported by Shahid Beheshti University of Medical sciences.

Conflict of interests
The authors declare that there is no conflict of interest.

References
1. Stadtlander CT, Waterbor JW. Molecular epidemiology, pathogenesis and prevention of gastric cancer. Carcinogenesis 1999; 20:2195-208.
2. Hong SJ, Kim SW. Helicobacter pylori Infection in Gastroesophageal Reflux Disease in the Asian Countries. Gastroenterol Res Pract 2015; 2015:985249.
3. Graham DY, Malaty HM, Evans DG, Evans DJ Jr, Klein PD, Adam E. Epidemiology of Helicobacter Pylori in an asymptomatic population the United States. Gastroenterology 1991;100:1495-501.
4. Kazemian H, Shavaliipour A, Mohebi R, Ghafurian S, Aslani A, Maleki A, et al. Estimation of the Parasitic Infection Prevalence in Children With Helicobacter pylori Infection in Ilam City (2012-2013). Pediatr Infect Dis J 2014;2:e15294.
5. Tomer Y, Davies TF. Infection, thyroid disease, and autoimmunity. Endocr Rev 1993;14:107-20.
6. Vaezi MF, Swoger J. Gastro-oesophageal reflux disease in the elderly. In: Grandrath, FA, Pointner KT, eds. Gastro-oesophageal Reflux Disease. Springer Vienna 2006;23-45.
7. Erzin Y, Koksal V, Altun S, Dobrucali A, Aslan M, Erdamar S, et al. Prevalence of Helicobacter pylori vacA, cagA, cagE, iceA, babA2 genotypes and correlation with clinical outcome in Turkish patients with dyspepsia. Helicobacter 2006;11(6):574-80.
8. Corley DA, Kubo A, Levin TR, Block G, Habel L, Rumore G, et al. Helicobacter pylori and gastroesophageal reflux disease: a case-control study. Helicobacter 2008;13:352-60.
9. Gudlaugsdottir S, Van Dekken H, Stijnen T, Wilson JH. Prolonged use of proton pump inhibitors, CagAstatus, and the outcome of Helicobacter pylori gastritis. J Clin Gastroenterol 2002;34:536-40.
10. Mansour-Ghanaei F, Joukar F, Atshani SM, Chagharvand S, Souti F. The epidemiology of gastroesophageal reflux disease: a survey on the prevalence and the associated factors in a random sample of the general population in the Northern part of Iran. Int J Mol Epidemiol Genet 2013;4:175-82.
11. Dabiri H, Maleknjad P, Yamaoka Y, Feizabadi MM, Jafari F, Rezadehbashi M, et al. Distribution of Helicobacter pylori cagA, cagE, oipA and vacA in different major ethnic groups in Tehran, Iran. J Gastroenterol Hepatol 2009;24:1380-86.
12. Rokkas T, Ladas SD, Triantafyllou K, Liatsos C, Petridou E, Papatheodorou G, et al. The association between CagA status and the development of esophagitis after the eradication of Helicobacter pylori. Am J Med 2001;110:703-7.
13. Take S, Mizuno M, Ishiki K, Nagahara Y, Yoshida T, Yokota K, et al. Helicobacter pylori eradication may induce de novo, but transient and mild, reflux esophagitis: prospective endoscopic evaluation. J Gastroenterol Hepatol 2009;24:107-13.
14. Cremonini F, Di Caro S, Delgado-Aros S, Sepulveda A, Gasbarrini G, Gasbarrini A, et al. Meta-analysis: the relationship between Helicobacter pylorinfection and gastrooesophageal reflux disease. Aliment Pharmacol Ther 2003;18:279-89.
15. Shin WG, Kim HU, Kim SG, Kim GH, Shim KN, Kim JW, et al. Work productivity and activity impairment in gastroesophageal reflux disease in Korean full-time employees: a multicentre study. Dig Liver Dis 2012;44:286-91.
16. Quigley EM, Hungin AP. Review article: quality-of-life issues in gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2005;1;41-7.
17. Dean BB, Crawley JA, Schmitt CM, Wong J, Ofman JJ. The burden of illness of gastro-oesophageal reflux disease:impact on work productivity. Aliment Pharmacol Ther 2003;15;17:1309-17.
18. Azuma T, Yamazaki S, Yamakawa A, Ohtani M, Muramatsu A, Suto H, et al. Association between diversity in the Src homology 2 domain—containing tyrosine phosphatase binding site of Helicobacter pylori CagA protein and gastric atrophy and cancer. J Infect Dis 2004;1;189:820-7.
19. Grande M, Lisi G, De Sanctis F, Grande S, Esser A, Campanelli M, et al. Does a relationship still exist between gastroesophageal reflux and Helicobacter pylori in patients with reflux symptoms?. World J Surg Oncol 2014;12:375.
20. Mahdi BM. The relationship between helicobacter pylori infection and gastro-esophageal reflux disease. N Am J Med Sci 2011;3:142-5.
21. Johnson LF, DeMeester TR. Development of the 24-hour intraesophageal pH monitoring composite scoring system. J Clin Gastroenterol 1986;8:52-8.
22. Smout AJPM. Endoscopy-negative acid reflux disease. Aliment Pharmacol Ther 1997;11:81-5.
23. Gisbert JP, Pajares JM, Losa C. Helicobacter pylori and gastroesophageal reflux disease: friends or foes?. Hepatogastroenterology 1999;46:1023-9.
24. Koike T, Ohara S, Sekine H, Iijima K, Kato K, Shimosegawa T, et al. Helicobacter pylori infection inhibits reflux esophagitis by inducing atrophic gastritis. Am J Gastroenterol 1999;94:3468-72.
25. Koike T, Ohara S, Sekine H, Iijima K, Abe Y, Kato K, et al. Helicobacter pylori infection prevents erosive reflux oesophagitis by decreasing gastric acid secretion. Gut 2001;49:330-4.

26. Dent J, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut 2005;54:710-7.

27. Becher A, Dent J. Systematic review: ageing and gastrooesophageal reflux disease symptoms, oesophageal function and reflux oesophagitis. Aliment Pharmacol Ther 2001;33:442-54.

28. Kim N, Lee SW, Cho SI, Park CG, Yang CH, Kim HS, et al. The prevalence of and risk factors for erosive oesophagitis and non-erosive reflux disease: a nationwide multicentre prospective study in Korea. Aliment Pharmacol Ther 2008;27:173-85.

29. Wong WM, Lai KC, Lam KF, Hui WM, Hu WH, Lam CL, et al. Prevalence, clinical spectrum and health care utilization of gastro-oesophageal reflux disease in a Chinese population: a population-based study. Aliment Pharmacol Ther 2003;18:595-604.

30. Chiba H, Gunji T, Sato H, Iijima K, Fujibayashi K, Okumura M, et al. A cross-sectional study on the risk factors for erosive esophagitis in young adults. Intern Med 2012;51:1293-9.

31. Ashktorab H, Entezari O, Nouraie M, Dowlati E, Frederick W, Woods A, et al. Helicobacter pylori protection against reflux esophagitis. Dig Dis Sci 2012;57:2924-8.

32. Hamada H, Haruma K, Mihara M, Kamada T, Yoshihara M, Sumii K, et al. High incidence of reflux oesophagitis after eradication therapy for Helicobacter pylori: impacts of hiatal hernia and corpus gastritis. Aliment Pharmacol Ther 2000;14:729-35.

33. Rokkas T, Ladas SD, Triantafyllou K, Liatsos C, Petridou E, Papatheodorou G, et al. The association between CagA status and the development of esophagitis after the eradication of Helicobacter pylori. Am J Med 2001;110:703-7.

34. Xie T, Cui X, Zheng H, Chen D, He L, Jiang B. Metaanalysis: eradication of Helicobacter pylori infection is associated with the development of endoscopic gastroesophageal reflux disease. Eur J Gastroenterol Hepatol 2013;25:1195-205.

35. Rubenstein JH, Inadomi JM, Scheiman J, Schoenfeld P, Appelman H, Zhang M, et al. Association between Helicobacter pylori and Barrett’s Esophagus, Erosive Esophagitis, and Gastroesophageal Reflux Symptoms. Clin Gastroenterol Hepatol 2014;12:239-45.

36. Yañez MA, Barberá VM, Soria E, Catalán V. Quantitative detection of Helicobacter pylori in water samples by real-time PCR amplification of the cag pathogenicity island gene, cagE. J Appl Microbiol 2009;107:416-24.

37. Patel SK, Pratap CB, Jain AK, Gulati AK, Nath G. Diagnosis of Helicobacter pylori: What should be the gold standard? World J Gastroenterol 2014;20(36):12847-59.

38. Mégraud F. Advantages and disadvantages of current diagnostic tests for the detection of Helicobacter pylori. Scand J Gastroenterol Suppl 1996;215:57-62.

39. Patel SK, Mishra GN, Pratap CB, Jain AK, Nath G. Helicobacter pylori is not eradicated after triple therapy: a nested PCR based study. Biomed Res Int 2014;2014:483136.

40. Lima VP, Rabenhorst SHB. Genes Associados à Virulência de Helicobacter pylori. Rev Bras Cancerologia 2009;55:389-96.

41. Ramis IB, Vianna JS, Silva Junior LV, Von Groll A, Silva PE. cagE as a biomarker of the pathogenicity of Helicobacter pylori. Rev Soc Bras Med Trop 2013;46:185-9.

42. Price AB. The Sydney System: histological division. J Gastroenterol Hepatol 1991;16:209-22.

43. Godoy AP, Ribeiro ML, Benvengo YH, Vitiello L, Miranda Mde C, Mendonça S, et al. Analysis of antimicrobial susceptibility and virulence factors in Helicobacter pylori clinical isolates. BMC Gastroenterol 2003;3:20.