Diversity of *Helicobacter pylori* genotypes in Iranian patients with different gastroduodenal disorders

Farzam Vaziri, Shahin Najar Peerayeh, Masoud Alebouyeh, Tabassom Mirzaei, Yoshio Yamaoka, Mahsa Molaei, Nader Maghsoudi, Mohammad Reza Zali

**Abstract**

**AIM:** To investigate the diversity of *Helicobacter pylori* (H. pylori) genotypes and correlations with disease outcomes in an Iranian population with different gastroduodenal disorders.

**METHODS:** Isolates of *H. pylori* from patients with different gastroduodenal disorders were analyzed after culture and identification by phenotypic and genotypic methods. Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Germany). After DNA extraction, genotyping was done for cagA, vacA s and m regions, iceA (iceA1, iceA2) and babA with specific primers for each allele using polymerase chain reaction (PCR). All patients’ pathologic and clinical data and their relation with known genotypes were analyzed by using SPSS version 19.0 software. χ² test and Fisher’s exact test were used to assess relationships between categorical variables. The level of statistical significance was set at \( P < 0.05 \).

**RESULTS:** A total of 71 isolates from 177 patients with different gastroduodenal disorders were obtained. Based on analysis of the cagA gene (positive or negative), vacA s-region (s1 or s2), vacA m-region (m1 or m2), iceA allelic type (iceA1 and iceA2) and babA gene (positive or negative), twenty different genotypic combinations were recognized. The prevalence of cagA, vacA s1, vacA s2, vacA m1, vacA m2, iceA1, iceA2, iceA1+iceA2 and babA were 62%, 78.9%, 19.7%, 21.1%, 78.9%, 15.5%, 22.5%, 40.8% and 95.8%, respectively. Interestingly, evaluation of PCR results for cagA in 6 patients showed simultaneous existence of cagA variants according to their size diversities that proposed mixed infection in these patients. The most prevalent genotype in cagA-positive isolates was cagA\(^+/\)vacA\(s1m1\)/iceA1+iceA2/babA+ and in cagA-negative isolates was cagA\(^-/\)vacA\(s2m2\)/iceA-/babA+. There were no relationships between the studied genes and histo-

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pathological findings (H. pylori density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy). The strains which carry cagA, vacAs/m, iceA and babA genes showed significant associations with severe active chronic gastritis ($P = 0.011, 0.025, 0.020$ and $0.031$, respectively). The vacAs genotype had significant correlation with the presence of the cagA gene ($P = 0.013$). Also, babA genotype showed associations with cagA ($P = 0.024$). In the combined genotypes, only cagA$^+$/vacAs$^+$/iceA$^+$/babA$^+$ genotype showed correlation with severe active chronic gastritis ($P = 0.025$).

CONCLUSION: This genotyping panel can be a useful tool for detection of virulent H. pylori isolates and can provide valuable guidance for prediction of the clinical outcomes.

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Key words: Helicobacter pylori; cagA; vacA; iceA; babA

INTRODUCTION

Infection with Helicobacter pylori (H. pylori) causes different clinical disorders such as persistent gastritis, peptic ulcers and mucosa associated lymphoid tissue (MALT) lymphoma. Current studies suggest that H. pylori infection may be a crucial risk factor in the development of gastric cancer[1-3]. In this regard, this pathogen has been categorized as a group I carcinogen by the International Agency for Research on Cancer[4]. The detailed reasons for these different clinical outcomes are unknown, but they may be related to host genetic factors, exposure to environmental factors (e.g., diet, drug usage, acidity of the stomach and smoking) and to the bacterial genotypes[5]. H. pylori shows extensive genetic diversity and this variability has a crucial role in pathogenesis of this bacterium[6]. Several H. pylori virulence factor genes related to the risk of gastroduodenal disorders, including cagA, vacA, babA and iceA, have been described[6]. A tremendous number of studies have proved that CagA and VacA producing strains are related to severe clinical outcomes[7]. In addition to cagA and vacA, the other H. pylori virulence factors, such as iceA and babA, also showed such associations in some studies[8]. Beyond the role of these factors in progression of the disease, there are several papers which reported a relationship between failure of H. pylori eradication therapy and the strains’ virulence factor genotypes[9]. Analysis of genetic structure of virulence factors among the isolates from different geographic regions will provide new insights regarding the pathogenesis and treatment of H. pylori infection. H. pylori genotyping may have multiple roles including impact on the cure rates of eradication therapy[10], determination of clinical outcomes[11], tracking human migration[12,13] and recently, the prediction of progression of gastric precancerous lesions[14]. The distribution pattern of H. pylori genotypes and its correlation with disease outcome shows geographic differences. The aim of this study was to assess the diversity of H. pylori genotypes in an Iranian population to determine genotypically the H. pylori isolates more associated with different gastroduodenal disorders.

MATERIALS AND METHODS

Clinical specimens

Three gastric biopsies (two were used for histological examination and one for culture) were obtained from 177 adult patients undergoing routine diagnostic endoscopy referred to the Endoscopy Centre of Taleghani Hospital of Tehran, Iran, after obtaining informed consent. All subjects answered questionnaires related to age, sex, gastroduodenal peptic ulcer disorders upon endoscopy.

Culture

Antral or body biopsy specimens from each patient were kept in transport medium consisting of thiglycollate with 1.5 g/L agar (Merek) and 3% yeast extract (Oxoid). The endoscopic biopsy specimens were cut into small pieces, homogenized with a sterile scalpel and were smeared on the surface of Brucella agar plates supplemented with 7% horse blood and Campylobacter selective supplement (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg) and amphotericin B (2.5 mg/L). Incubation was performed in microaerophilic conditions at 37 °C for 5-7 d. Identification of H. pylori isolates was performed by analyzing colony morphology, Gram staining, oxidase, catalase and urease activities and H. pylori-specific polymerase chain reaction (PCR) (glmM). The isolates were preserved in BHI broth containing 20% glycerol and 10% fetal calf serum and stored at -70 °C.

DNA extraction

Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer’s instructions. The DNA was stored at -20 °C until used for molecular studies.

H. pylori genotyping

After DNA extraction, polymerase chain reactions (PCR) were performed in a volume of 25 μL containing 1 × PCR buffer, 1 μmol/L of each primer, 1 μL of genomic DNA (approximately 150 ng), 200 μmol/L of dNTPs mix, 2 mmol/L of MgCl$_2$, and 0.05 U/μL Taq DNA polymerase. PCR amplifications were performed in an automated thermal cycler (Ag 22331; Eppendorf, Hamburg, Germany) under the following conditions: for vacA $s/m$ 33 cycles of 1 min at 94 °C, 33 s at 55 °C, and 1 min at 72 °C; for cagA: 33 cycles of 1 min at 94 °C, 1 min at
59°C, and 1 min at 72°C; for vacA s/m: 33 cycles of 1 min at 94°C, 40 s at 58°C, and 1 min at 72°C, and for babA: 35 cycles of 1 min at 94°C, 40 s at 58°C and 1 min at 72°C. The amplified genes were detected by electrophoresis in a 1.2% agarose gel with ethidium bromide. Table 1 summarizes the primer sequences, annealing temperatures and the expected size of the PCR products.

Histopathological evaluation
Sections were stained with hematoxylin and eosin for analysis of H. pylori-related histology by an expert pathologist. Then the grade of gastritis was scored based on the updated Sydney System.

Statistical analysis
Data were analyzed by using SPSS version 19.0.0 software (IBM, IL, United States). χ² test and Fisher’s exact test were used to assess relationships between categorical variables.

RESULTS

Infection rates and clinical disorders
A total of 71 isolates from 177 patients (parenthesis approximately 40%) with different gastroduodenal disorders were obtained. The H. pylori-positive patients consisted of 24 males and 47 females, with their ages ranging between 19 and 85 years (mean age, 66 years). All of the isolates showed positive results for the common identification test and H. pylori-specific PCR (glmM). Most of the infected patients suffered from chronic gastritis (84.6%), while the others showed duodenitis (9.8%), intestinal metaplasia (2.8%), hyperplasia (1.4%) and gastric cancer diseases (1.4%) (Table 2).

Allelic diversities in main putative virulence markers

cagA genotyping: The 400-bp PCR product indicating the presence of the cagA gene was obtained in 44 isolates (62%) and 27 (38%) were negative. Interestingly, evaluation of PCR results for cagA in 6 patients showed simultaneous existence of cagA variants according to their size diversities.

vacA genotyping: The frequency of vacA s, vacA s, vacA m and vacA m were 78.9%, 19.7%, 21.1% and 78.9%, respectively. Only one isolate was vacA s (with no PCR product for s region).

iceA genotyping: Sole existence of iceA1F genotype was detected in 15.5% and iceA2 F genotype in 22.5% of the colonized patients. Interestingly, out of the total studied samples, 40.8% were infected with both iceA and iceA1F genotypes and 21.1% were negative for these genes.

babA genotyping: babA was found in 68 of the patients (95.8%); however, three patients (4.2%) did not show this allelic variant (Figure 1).

Correlation of H. pylori genotypes with pathological data, patients’ age and clinical outcome

Combination of genotypes: Based on the analysis of the cagA gene (positive or negative), vacA s-region (s or s), vacA m-region (m or m), iceA allelic types (iceA1 and iceA2) and babA (positive or negative), twenty different genotypic combinations were recognized. The most prevalent genotype in cagA positive isolates was cagA1/vacA s/m/iceA+/babA+ and in cagA negative isolates was cagA0/vacA s/m/iceA+/babA+ (Figure 2).

Helicobacter pylori density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy: There was no significant relationship between cagA positivity and H. pylori density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy in the biopsies. Also no relationships were found between other genes and these histopathological findings.

Patients’ age: There was no significant relationship between the genotypes, clinical and pathological data and patients’ age.

Chronic gastritis: The gastritis was scored as severe ac-
Table 2  Association of combined genotypes with pathological conditions in *Helicobacter pylori* isolates

| Combination of genotypes | SCG | SACG | MACG | MIAACG | MCG | H | M | GC | D | Total | P value |
|--------------------------|-----|------|------|--------|-----|---|---|----|---|-------|---------|
| cagA+/vacAs1m2/iceA1+iceA2/babA+ | 1   | 2   | 0   | 0     | 0   | 1 | 0 | 1  | 17|       | 0.025   |
| cagA+/vacAs1m1/iceA2/babA+   | 0   | 3   | 0   | 0     | 0   | 0 | 0 | 1  | 4 |       | 0.013   |
| cagA+/vacAs1m1/iceA1/babA+   | 0   | 3   | 0   | 0     | 0   | 1 | 0 | 2  | 7 |       | 0.011   |
| cagA+/vacAs1m1/iceA1+iceA2/babA+ | 0   | 6   | 1   | 0     | 1   | 0 | 0 | 0  | 8 |       | 0.024   |
| cagA+/vacAs2m1/iceA1+iceA2/babA+ | 0   | 1   | 1   | 0     | 0   | 0 | 0 | 0  | 2 |       | 0.013   |
| cagA+/vacAs1m2/iceA2/babA+   | 0   | 0   | 0   | 0     | 0   | 0 | 0 | 1  | 1 |       | 0.005   |
| cagA+/vacAs1m1/iceA1/babA+   | 0   | 0   | 0   | 0     | 0   | 0 | 0 | 0  | 1 |       | 0.005   |
| cagA+/vacAs1m2/iceA1+babA+   | 0   | 1   | 0   | 0     | 0   | 0 | 0 | 0  | 1 |       | 0.025   |
| cagA+/vacAs1m1/iceA1+babA+   | 0   | 1   | 0   | 0     | 0   | 0 | 0 | 0  | 1 |       | 0.025   |
| cagA+/vacAs1m1/iceA1+babA+   | 0   | 3   | 0   | 0     | 0   | 0 | 0 | 0  | 4 |       | 0.025   |
| cagA+/vacAs1m2/iceA1+babA+   | 0   | 1   | 0   | 0     | 0   | 0 | 0 | 0  | 1 |       | 0.025   |
| cagA+/vacAs1m2/iceA1+babA+   | 0   | 3   | 0   | 0     | 0   | 0 | 0 | 0  | 4 |       | 0.025   |
| cagA+/vacAs1m2/iceA1+babA+   | 0   | 0   | 0   | 1     | 0   | 0 | 0 | 0  | 1 |       | 0.025   |
| cagA+/vacAs1m2/iceA1+babA+   | 0   | 0   | 1   | 0     | 0   | 0 | 0 | 0  | 2 |       | 0.025   |
| Total                     | 2   | 39  | 13  | 1     | 5   | 1 | 2 | 1  | 71|       | 0.024   |

1Only P < 0.05 are indicated; 2This P value is related to severe active chronic gastritis (SACG). SCG: Severe chronic gastritis; MACG: Moderate active chronic gastritis; MIAACG: Mild active chronic gastritis; MCG: Moderate chronic gastritis; H: Hyperplasia; M: Metaplasia; GC: Gastric cancer; D: Duodenitis.

DISCUSSION

*Helicobacter pylori* infection is usually present in 60%-80% of gastric and 95% of duodenal ulcers. However, some conditions affect infection rate of this bacterium in different geographic and socioeconomic regions. The prevalence of infection is typically higher in developing countries (greater than 80%) and lower in the developed ones (typically less than 40%) [19]. It has been demonstrated that the prevalence of *H. pylori* infection in developing countries with low socioeconomic status and poor management of drinking water is much higher (> 80%) than that in developed countries (< 60%) [20]. In our study the recovery rate of *H. pylori* was 40% which shows the improvement in the living conditions and hygiene in Iran that has also been reported recently [21].

*H. pylori* can be divided into *cagA*-positive and *cagA*-negative strains, and there is increasing evidence that infection with *cagA*-positive isolates is associated with a greater risk of adverse clinical outcomes than infections with strains lacking this gene. In the current study, the strains which carried the *cagA* gene showed significant associations with severe active chronic gastritis (P = 0.011). Also, the strains which carried the *vacA S1/S1* genotype showed significant associations with severe active chronic gastritis (P = 0.025), *babA* (P = 0.031) and *iceA1* (P = 0.020) also had significant correlation with severe active chronic gastritis. In the combined genotypes this association was observed for *cagA*+/*vacAs1m1/iceA1+babA+* genotype in the case of severe active chronic gastritis (P = 0.025).

Genotype correlation: Interestingly, the *vacA S1* genotype had significant correlation with the presence of the *cagA* gene (P = 0.013). Also *babA* genotype showed this association in *cagA* positive isolates (P = 0.024).
In our study the prevalence of \textit{cagA}-positive isolates is 62\% which is less than other Asian countries and more than other countries (e.g., Ecuador, Panama). According to Watada \textit{et al.}\cite{25} study, the prevalence of \textit{cagA} was 65.5\% in Colombia and 100\% in Japan, which showed that the prevalence of this gene in our study is similar to the Colombian isolates. In another study conducted in Bulgaria, the prevalence of \textit{cagA} was 84.9\% which is more than our results\cite{26}. Interestingly, we had 6 isolates which had two different sizes of \textit{cagA} simultaneously, showing the occurrence of mixed infection in these patients.

Variations of \textit{vacA} are associated with different risks of gastrointestinal disorders. In general, \textit{vacA} \textit{s} and \textit{m} genotypes produce a large amount of toxin, whereas \textit{s}\textsubscript{1} and \textit{m}\textsubscript{2} genotypes show little or no toxin production\cite{27}. Recently, a third polymorphic determinant of vacuolating activity has been described as located between the \textit{s}-region and \textit{m}-region, an intermediate (i) region\cite{28}. The frequency of the \textit{vacA s} and \textit{vacA m} genotypes in the Middle Eastern countries was found to be 71.5\% and 32.8\%, respectively\cite{29}, which is in concordance with our study. We did not detect any \textit{vacA s\textsubscript{0}m\textsubscript{2}} genotypes in our isolates which has been reported to be rare\cite{29}. The \textit{vacA s\textsubscript{1}} and \textit{m}\textsubscript{1} genotypes have been reported to be associated with \textit{H. pylori}-related diseases; however \textit{vacA s\textsubscript{2}} and \textit{m}\textsubscript{2} strains are rarely associated with peptic ulcer and gastric cancer because of their low or non-vacuolating activities\cite{29}. Genotyping of \textit{vacA} will be useful in screening individuals for risk factors associated with gastric cancer and peptic ulcer development. Asrat \textit{et al.}\cite{30} showed that \textit{vacAs1m1} genotype was the most common genotype in Ethiopian adult dyspeptic patients, and also \textit{vacA}- and \textit{cagA}-positive \textit{H. pylori} strains were detected to a higher degree in patients with chronic active gastritis. Interestingly, similar to our results, correlation of the \textit{vacA s} genotype with the presence of the \textit{cagA} gene was reported by Atherton\cite{31}. The \textit{vacAs1m2} genotype is more common in our Iranian patients, as previously described in Iran\cite{31}. As reviewed by Suzuki \textit{et al.}\cite{32}, the predominant \textit{vacA} genotypes in Asia, Europe and Africa are \textit{vacAs1m1} and their subtypes, which is in contrast to our genotypes in Iranian isolates.

In spite of the low frequency of \textit{vacAs1m1} genotypes in our study, isolates which carried the \textit{vacAs1m1} gene showed significant associations with severe active chronic

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**Figure 2** The frequency of combined genotypes. A: Combined \textit{vacA}, \textit{iceA} and \textit{babA} genotypes in 44 \textit{cagA} positive isolate; B: Combined \textit{vacA}, \textit{iceA} and \textit{babA} genotypes in 27 \textit{cagA} negative isolates.
In a review by Hosseini et al.\(^\text{[7]}\), they concluded that in contrast to vacA, there is no correlation between cagA genotype and disease status in the majority of studies conducted in Iran; but results of our study, however, proposed both of these genetic markers as useful indicators for predicting clinical outcomes in the studied population.

The meta-analysis by Shiota et al.\(^\text{[8]}\) confirmed the importance of the presence of iceA gene for peptic ulcer, although the significance was controversial. Such different results between the iceA allelic types and clinical disorders could be explained by the difference in geographic regions. In our study we found a significant relationship between iceA: genotype and clinical outcomes (severe active chronic gastritis), which was also observed by Caner et al.\(^\text{[9]}\) in Turkey. As Shiota et al.\(^\text{[8]}\) summarized in their meta-analysis, most of the studies showed no association between iceA: and cagA status, which is in concordance with our study. Interestingly, the prevalence of mixed genotype iceA:1 + iceA:2 (40.8%) in our study was higher than other studies which had detected this mixed genotype\(^\text{[10-12]}\). So this high prevalence with mixed genotypes makes it difficult to analyze potential relationships between the presence of each iceA allelic variant and clinical outcomes. babA genotype was frequently found in H. pylori strains in our study (95.8%); this was associated with severe active chronic gastritis. Although this genotype showed significant correlation with the existence of cagA, no significant correlation was observed with other virulence factors such as vacA s/s, vacA m1/m1 and iceA/iceA. Chomvarin et al.\(^\text{[38]}\) detected the babA gene in 92% (103/112) of Thai patients, which is almost similar to our results; while in another study conducted in Cuba the prevalence of babA gene was lower (82.3%)\(^\text{[39]}\). It is important to mention that this PCR based method for babA genotyping must be confirmed by immunoblotting. Actually isolates were scored as babA:gene positive if the PCR and/or Southern blot analysis yielded a positive result\(^\text{[38]}\).

Regarding the combination of genotypes, we observed twenty different genotypes which showed vast diversities in the H. pylori isolates in our study. Interestingly there was not any significant association between these combined genotypes and clinical outcomes, except for cagA+/vacA:sm/iceA2/babA+ genotype which showed significant association with severe active chronic gastritis.

Genotypes of H. pylori, especially cagA and vacA, are reported to be crucial factors determining the cure rates. So to select an H. pylori eradication regimen, we need to consider H. pylori genotypes\(^\text{[39]}\). H. pylori genotype distributions and their correlations with disease outcomes have shown geographical differences. In this regard, Yamaoka et al.\(^\text{[39]}\) reviewed that within East Asia, where the incidence of gastric cancer is high, that vacA m1 genotype is dominant; whereas in southern parts where the gastric cancer incidence is low, the m2 genotype, which we observed in our study, is predominant. Dabiri et al.\(^\text{[40]}\) showed that there was statistically no association between the vacA, cagA and cagE: status and clinical outcomes in Iranian patients, and recommended that other different markers may be more useful for this analysis. In comparison, in the current study, genotyping on the basis of cagA, babA, vacA and iceA was considered as a useful tool for predicting the clinical outcomes. Therefore, analyzing the multiple virulence factors of H. pylori (cagA, vacA, iceA and babA) might enable us to predict the patient’s clinical outcome among Iranian patients. This prediction could be more accurate when accompanied by the impacts of environmental factors and host genetic polymorphisms such as interleukin-1 receptor antagonist gene polymorphism\(^\text{[39]}\). Nowadays, concurrent genotyping of H. pylori virulence markers and host factors is becoming increasingly crucial in the prediction of the diseases outcomes\(^\text{[40]}\).

In conclusion, our results show that most of the H. pylori isolates were highly virulent on the basis of the main clinically allelic variants in three or four virulence factors they carried. The Iranian isolates predominantly possessed different genotypes which showed vast diversities. Significant association of the noted genotypes with severe active chronic gastritis suggests that this genotyping panel is a suitable tool for detection of virulent H. pylori isolates that could provide valuable guidance for prediction of the clinical outcomes.

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**COMMENTS**

**Background**

Infection with Helicobacter pylori (H. pylori) causes diverse clinical outcomes such as persistent gastritis, peptic ulcers, mucosa associated lymphoid tissue lymphoma and gastric cancer. One of the reasons for these different clinical outcomes is genetic diversity of H. pylori; therefore determination of the pattern of H. pylori genotypes and its correlation with disease outcome, which shows geographic differences, is crucial.

**Research frontiers**

The H. pylori genotyping may have multiple roles including prediction of clinical outcomes, impact on the H. pylori infection therapy, tracking human migration, and recently, the prediction of progression of gastric preneoplastic lesions. Therefore genotyping of H. pylori can be a valuable and multifunctional tool in the clinical field.

**Innovations and breakthroughs**

In the majority of previous studies, the researchers were not able to detect any significant relationship between their genotyping panels and clinical outcomes for H. pylori infections. Most of these studies had used few genetic markers. In order to overcome this disadvantage, the authors have chosen greater numbers of H. pylori genetic markers for studying this association.

**Applications**

The genotyping panel which contains eight important genetic markers can be more accurate when accompanied by the impacts of environmental factors and host genetic polymorphisms such as interleukin-1 receptor antagonist gene polymorphism\(^\text{[39]}\). Nowadays, concurrent genotyping of H. pylori virulence markers and host factors is becoming increasingly crucial in the prediction of the diseases outcomes\(^\text{[40]}\).

**Peer review**

This is an epidemiological paper with statistical analysis, dealing with the important question of association between certain H. pylori genotypes and specific
pathologies, and with the problem of predictive value of H. pylori infection genotyping. In the submitted manuscript this issue is dissected in fine detail and uses quite extensive clinical material, thus providing novel and more reliable data.

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