Relationship Between Drug Resistance and cagA Gene in *Helicobacter pylori*

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Background: Currently, there are evidences for the existence of diverse strains of *Helicobacter pylori* with different levels of virulence. In other way, the most important reason of therapeutic failure is resistance to the antibiotics.

Objectives: The aim of this study was to evaluate the relationship between *H. pylori* drug resistance and the presence of cagA gene.

Patients and Methods: Gastric biopsy specimens were cultured for *H. pylori* isolation. Resistance to antimicrobial agents, including clarithromycin, metronidazole, and amoxicillin were detected by modified disk agar diffusion tests. The cagA gene was detected using PCR, and the results were analyzed by $\chi 2$ and Fishers exact tests using SPSS software version 16.

Results: A total of 123 *H. pylori*-positive patients (68 males, 55 females, mean age 35 ± 18 years old) were studied. The rate of resistance to clarithromycin, metronidazole and amoxicillin were 17.07, 78.86 and 27.64%, respectively. In this study, the cagA was detected in 84 of 123 strains (68.3%). The frequency of resistance to clarithromycin, metronidazole and amoxicillin in cagA positive confirmed microorganisms were, 16 (13.01%), 67 (54.47%) and 24 (19.51%), respectively. While the results for cagA negative ones were 5 (4.06%), 30 (24.39%) and 10 (8.13%), respectively.

Conclusions: Our results indicated that the overall antibiotic resistance of *H. pylori* was high, and the relationship between drug resistance and cagA status was not statistically significant. Therefore, different treatment regimens are not recommended for patients with *H. pylori*-cagA positive infections.

Keywords: Antimicrobial Resistance; cagA Gene; *Helicobacter pylori*

1. Background

Helicobacter pylori (*H. pylori*) is a microaerophilic spiral-shaped Gram-negative bacterium, which is found in the gastric mucous layer or adherent to the epithelial lining of the stomach (1). The prevalence of *H. pylori* infection is reported as approximately 50%, worldwide, which is as high as 80%-90% in developing countries (2). Although most infected persons remain asymptomatic, but 15-20% of *H. pylori*- positive individuals will develop the associated diseases. The occurrence of diverse diseases as peptic ulcer, gastric cancer and MALT lymphoma with *H. pylori* depends on specific features of the organism, host genetic and environmental factors (3).

H. pylori express the virulence factors that augment the risk of clinical disease outcomes. The virulence factor that has attracted most of the interests is the cytotoxin-associated gene A (cagA), which encodes the CagA protein and the cellular effects of CagA may clarify why patients infected with cagA strains, usually have higher inflammatory responses and level of proinflammatory cytokines. Thus, these gastric mucosal responses induce the peptic ulcer diseases, precancerous lesions, and cancer in adults (4). Approximately 60% of *H. pylori* strains isolated in Western countries carry cag pathogenesity island (cag PAI), whereas almost all of the isolates from East Asia are cag PAI-positive (5).

The Maastricht Ⅲ consensus advised that proton pump inhibitors (PPI), plus clarithromycin, amoxicillin or metronidazole therapy for 7 to 14 days as the first choice for *H. pylori* infection management (6). Recently, several large clinical trials and meta-analyses have shown that the eradication of the standard triple therapy has gener-

ally declined to unacceptable levels (i.e., 80% or less) (7). Resistance to antibiotics is the chief factor for treatment failure (2). In other way, results of some studies have suggested that the eradication rate in patients with gastritis is lower than that the patients with peptic ulcer diseases (8). Since the worldwide increase of the drug resistance rates represents a problem of relevance, some researches have been conducted on antibiotic resistance relationship with bacterial genetic factors (9, 10). If harboring cagA affects the response rate of therapy, then it may be possible to anticipate *H. pylori* eradication rates.

2. Objectives

The aim of this research was to analyze the relationship between the antibiotic resistance and cagA presence in *H. pylori* isolates

3. Patients and Methods

3.1. Patients

One hundred and twenty three *H. pylori* isolates were recovered from gastric biopsy samples of patients with gastritis, peptic ulcer and gastroesophageal reflux diseases underwent endoscopy in Azerbaijan, Iran. At least 1 week prior to endoscopy the patients had stopped receiving antimicrobial agent.

3.2. Bacterial Isolates

For bacterial culture, the gastric biopsy samples were homogenized and cultured onto the Brucella agar (Pronadisa Co, Spain) containing 5% sheep blood and antibiotics supplement (vancomycin 6 μg/mL, amphotricin B 2.5 μg/mL and trimethoprim 20 μg/mL). The cultured plates were incubated under microaerophilic condition (Mart, Anoxomat, Lichtenvoorde, Netherlands O_2 5%, CO_2

10%) at 37°C with high humidity for 5 - 7 days. *H. pylori* was detected based on colony morphology, by Gram staining and positive oxidase, catalase and urease tests.

3.3. Antibiotic Susceptibility Tests

Antimicrobial susceptibility of *H. pylori* isolates were determined by modified disk agar diffusion test. Suspensions of 3-day old cultures were prepared in sterile normal saline to match with No. 4 McFarland standard (11). The disks of antibiotics (MAST Co., England) including clarithromycin (15 μg), amoxicillin (25 μg), and metronidazole (5 μg) were placed on the inoculated plates and incubated for 72 hours. The inhibition zones were interpreted as previously reported (12).

3.4. DNA Extraction

Genomic DNA was extracted with the CTAB reagent method (13). The extracted DNA eluted in 50 μL of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) and stored at -20°C until use.

3.5. Assessment of cagA and glmM Genes by Multiplex PCR

Primer sequences (Table 1) and conditions of PCR amplifications of the *glmM* gene for the detection and confirmation of *H. pylori* and the *cagA* virulent gene were designed based on previous studies (14 , 15). Briefly, each PCR was performed in a total volume of 50 μL containing 100 ng *H. pylori* genomic DNA, 0.2 μL of dNTP (10mM), 0.2 μL PCR buffer (1X), 0.1 μL mgcl2 (1.5 mM), 0.2 μL of each primer and 1.5 μL of Taq polymerase (500 U). The PCR was carried out in an automated thermal cycler (Eppendorf, Germany). After amplification, 10 μL of PCR product was electrophoresed onto 1.5% agarose gel stained with ethidium bromide and later visualized under UV illuminator.

Table 1. The Primer Sequences Used in the Study

Gene	Primer	Nucleotide Sequence	Size (bp)
ureC (glmM)	HP-F	GGATAAGCTTTTAGGGGTGTTAGGGG	294
	HP-R	GCTTACTTTCTAACACTAACGCGC	
cagA	cagA-Fm	AGGGATAACAGGCAAGCTTTTGA	352
	cagA-Rm	CTGCAAAAGATTGTTTGGCAGA	

3.6. Statistical Analysis

The correlations between the *H. pylori*-cagA genotype and antibiotic susceptibilities were analyzed using $\chi 2$ test or Fishers exact test. A P value less than 0.05 was considered as statistically significant.

4. Results

The mean age of the patients was 35 ± 18 years. Out of 123

H. pylori isolates, 99 isolates were collected from adults, and 24 isolates from children. Overall, the rate of resistance to clarithromycin, metronidazole and amoxicillin were 17.07, 78.86 and 27.64%, respectively. Resistance to both metronidazole and clarithromycin were detected in 13% (n = 16). Of interest, 10 isolates (8.13%) were resistant to all antibiotics (clarithromycin, metronidazole and amoxicillin). The cagA gene was detected in 84 strains (68.3%) (Figure 1). The distribution of the cagA gene was the same

in both genders and age groups (73% children and 68% adults, 68.6% male and 68.8% female).

Figure 1. PCR Products of *glmM* and *cagA* Genes by Multiplex PCR
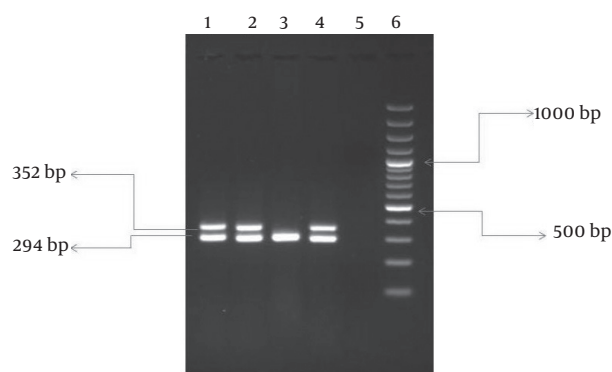
Lanes 1 and 2: patients 2 and 3 that were glmM and cagA positive, Lane 3: a patient sample with cagA negative and glmM positive, Lane 4: positive control, Lane 5: negative control, Lane 6: 100 bp DNA ladder.

The presence of cagA observed more likely in patients with peptic ulcer diseases (80%) than in non-ulcer dyspepsia ones (65%), and the difference was statistically significant (P value ≤ 0.05). The frequency of resistance to clarithromycin, metronidazole and amoxicillin in *H. pylori* - cagA positive were, 16 (13.01%), 67 (54.47%) and 24 (19.51%), respectively. While the frequency of resistance to these antibiotics in *H. pylori* - cagA negative were 5 (4.06%), 30 (24.39%) and 10 (8.13%), respectively. The distribution of cagA in different groups of gastroduodenal diseases and antibiotic resistance are demonstrated in Table 2.

5. Discussion

The three main antibiotics that are used as first-line eradication regimens against *H. pylori* are clarithromycin, metronidazole and amoxicillin (16, 17). However, *H. pylori* resistance to antimicrobials agents is the main cause of treatment failure (18). The level of resistance varies between different geographical regions and unfortunately, which is increasing worldwide. This study showed that the rate of resistance to metronidazole is high (78.86%). In developed countries, metronidazole resistance ranges from 10 to 50% while in developing counties, higher rate of resistance are reported (19). This is may be related to the greater consumption of this drug (2). In the case of

Table 2. Distribution of cagA in 123 *H. pylori* Strains Based on Diseases and Antibiotics Susceptibility Patterns

	cagA- Positive, No. (%)	cagA- Negative, No. (%)	Total, No.
Peptic ulcer diseases	16 (80)	4 (20)	20
Non-ulcer diseases	54 (65)	29 (34.9)	83
Am Resistance[a]	24 (19.51)	10 (8.13)	34
Cl Resistance[a]	16 (13.01)	5 (4.06)	21
Met Resistance[a]	67 (54.47)	30 (24.39)	97

[a] Abbreviations: Am, amoxicillin; Cl, clarithromycin; Met, metronidazole

clarithromycin, the second most commonly used antibiotic in *H. pylori* management, we found resistance rate as 17.07%. However, in Asian countries, a higher clarithromycin resistance rate has been reported in Japan (40.7%) while, the lowest value has been observed in Malaysia (2.1 %) (18). In North America, clarithromycin resistance ranges from 2.5 - 12.2 % (20).

In this research, the amoxicillin resistance was found in 27.64% of isolates. Until now, the resistance to amoxicillin in *H. pylori* was reported to be very rare; but have now been reported in USA (21), Italy (22), Brazil (23) and Iran (2, 24). Multiple drug resistance is a major problem worldwide. In the present study, we remarkably observed double resistance to clarithromycin and metronidazole and triple resistance to clarithromycin, metronidazole and amoxicillin. Pretreatment *H. pylori* antibiotic resistance has been reported to compromise the efficacy of management. For example, therapy regimens containing metronidazole and clarithromycin fail in as many as 38%

and 55% of cases, respectively, when used to treat infection with an organism non-susceptible to one of these antimicrobial agents (17).

There are new facts about the existence of various strains of *H. pylori* with different levels of virulence (1, 3, 8, 25-27). The cagA gene is located at one end of a 40-kilobase DNA section called cag pathogenicity island (cag PAI) (28, 29). The clinical relevance of the putative virulence- associated genes of *H. pylori* and geographical region is still a subject of controversy (28). The prevalence of cagA-positive *H. pylori* strains varies from one geographic region to another (29). In this research such as the study from Isfahan- Iran (30), the prevalence of cagA-positive *H. pylori* strains was 68.3% which is more similar to the findings reported from Western countries (18, 27) and Brazil (31) than those reported from East or Southeast Asia (14, 28, 32). The presence of the cagA gene is thought to be associated with a further severe clinical outcome of the disease (3, 27, 33-35). The current study demonstrated the

high prevalence of *H. pylori* infection containing cag A in patients from Azerbaijan with peptic ulcer disease than non-ulcer dyspepsia (Pv 0.02). Reports from Turkey (3) and Iraq (36) have shown similar results to our findings. Due to the allelic variation in cagA the *H. pylori* subtypes can circulate in different regions, the differences in cagA subtype might be a marker for different virulence among cagA positive *H. pylori* strains (32).

Furthermore, the severity of gastric inflammation and the pathology also affect the outcome of therapy (8, 9). There is now increasing evidences that cagA-positive strains induce an enhanced inflammatory response, mucosal damage and significantly delays healing of ulcers (1, 8). In this study, the rate of resistance to antibiotics in *H. pylori-* cagA positive were higher than cagA negative ones, however no statistically significant difference was found (P value > 0.05). Similarly, some studies also failed to find any general association between virulence markers and antibiotic resistance (18, 22, 37). This result is inconsistent with other studies that suggesting the statistically relation between drug resistance and cagA-positive strains (27, 31, 38, 39). There is little information about the relation between the variability of the strain and any outcome on the drug resistance rates of *H. pylori*. At the molecular level, there is no hypothetical basis for predicting an association between drug resistance and genotype; as such loci show to be neither physically nor functionally linked by genome organization (40).

Our research had some limitations. The studied population was not large enough, and our results need to be confirmed by studies on great number of patients. This study only tested the hypothesis that the cagA gene is related to antibiotic resistance. We think additional genetic or environmental factors might influence the interaction of *H. pylori* and antibiotics. In conclusion, the prevalence of *H. pylori* resistance is high in this area. Our results indicated that the relationship between drug resistance and the availability of cagA gene was not statistically significant. So, different treatment regimens are not recommended for patients with *H. pylori-* cagA positive infection.

Acknowledgements

Authors' Contribution

Financial Disclosure

Funding/Support

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