ORIGINAL RESEARCH

Clarithromycin-Susceptible But Virulent *Helicobacter pylori* Strains Infecting Iranian Patients' Stomachs

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Introduction: *Helicobacter pylori* was discovered first in the stomachs of patients with gastritis and ulcers by Marshall and Warren in 1982. This discovery majorly affected many research areas of gastroenterology. Since then, the main aim has been to eradicate this microaerophilic bacterium from the stomachs of infected subjects.

Methods: We studied symptomatic cases by endoscopic surgery and examined the prevalence of *cagA*–*vacA* genotypes among the *H. pylori* isolates. *H. pylori* isolated from antral biopsies of patients with gastritis and duodenal ulcer were subjected to antimicrobial susceptibility testing and PCR genotyping by using routine bacterial cultures. Clarithromycin-susceptibility profiling was done by the E-test. DNA was extracted using standard manufacturer protocols with minor modifications and *cagA* and *vacA* genotyping was done PCR.

Results: In our study, all strains identified as *H. pylori* in culture (61/81) were confirmed by PCR by amplifying a fragment of the *glmM* gene. Totally, 61 patients were confirmed to be positive for *H. pylori* and they were included in the genotyping and antibiotic-susceptibility testing. Thirteen *H. pylori* strains were determined to be resistant to clarithromycin.

Discussion: Current accumulating data indicate that both clarithromycin-resistant and susceptible isolates of *H. pylori* need to be screened and tracked in populations.

Keywords: antibiotic susceptibility, *cagA*, clarithromycin, E-test, *Helicobacter pylori*, genotypes, PCR, *vacA*

Introduction

Helicobacter pylori is a well-known bacteria, colonization of which inevitably causes gastroduodenal diseases, including chronic and atrophic gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma.[1–3] Because the incidence of digestive diseases associated with *H. pylori* can vary in different geographical regions, we may postulate that reported cases are dictated by other factors such as bacterial virulence. Virulent strains of *H. pylori* are generally accepted to cause severe digestive diseases and potentially genetically carry virulence factors.[4–6]

Furthermore, the discovery that peptic ulcers and acute gastritis can be treated efficiently by antibiotics was an important breakthrough in this century.[7,8] Therefore, finding the best effective antibiotic therapy for *H. pylori* isolates specific to various regions is highly desirable.[9] The recent Maastricht guideline was calling for treating all symptomatic *H. pylori*-infected individuals.[10] Broadly defined, three main reasons affect the efficacy of the recommended antibiotics therapies against this persistent bacterium; these include high bacterial load, poor patient compliance, and emergence of antibiotics resistance.[11] Among them, emergence of antibiotics

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resistance is an undeniable factor.[12,13] Proton-pump inhibitors (PPI)-clarithromycin containing triple therapy without prior susceptibility testing should be abandoned when the clarithromycin resistance rate in the region is more than 15%. In areas of high (>15%) clarithromycin resistance, bismuth quadruple or non-bismuth quadruple, concomitant (PPI, amoxicillin, clarithromycin and a nitroimidazole) therapies are recommended.[14] In areas of high dual clarithromycin and metronidazole resistance, bismuth quadruple therapy (BQT) is the recommended first-line treatment. In the case of allergic reactions, metronidazole can substitute amoxicillin. Moreover, clarithromycin resistance reportedly decreases the efficacy of therapy by more than 80%.[15–17] In reality, clarithromycin is a pivotal component in the treatment of *H. pylori* infection, but the probability of therapeutic failure is relatively high if the resistance rate is more than 15% in the population.[17,18] In addition, clarithromycin is readily available as a proposed treatment to eradicate *H. pylori* efficiently in symptomatic individuals.[19] The global resistance rate to clarithromycin has become alarming, and the World Health Organization (WHO) has warned against uncontrolled application of this key antibiotic under clinical settings.[20] The main purpose of our survey is to determine the prevalence of certain virulence factors (*cagA* and *vacA*) among clarithromycin-susceptible and clarithromycin-resistant *H. pylori* strains obtained from symptomatic Iranian patients and to investigate likely significant associations.

Materials And Methods
Sample Collection
This is a cross-sectional study conducted from August 2017 to August 2018. All patients, who were referred for upper gastrointestinal endoscopy at Imam-Hossein Hospital, Tehran, Iran, were enrolled. Three antral biopsy samples were taken from each patient for further analysis. The first biopsy sample was sent for histopathological examination; the second one was used for urease assay (Bahar-Azma, Tehran, Iran); and the last sample was placed in a small 1.5-mL tube containing the thioglycollate broth medium and kept at 4°C for bacterial culturing. The last biopsy samples were processed for bacterial culturing within 4 h of endoscopy. Exclusion criteria included age under 17 or over 80 years, severe cardiovascular diseases, antibiotic therapy against *H. pylori* 2 months before endoscopy, pregnancy, bleeding problems, history of abdominal

surgery a year before endoscopy, alcohol consumption 2 months before biopsy, and consumption of 1) nonsteroidal anti-inflammatory drugs 2) proton-pump inhibitors (PPI), or 3) bismuth a month before enrolment in the study. The study was first presented to, and approved by, the ethics committee of Tarbiat Modares University, Tehran, Iran (IR.TMU.REC. 1397.071), and all patients gave their written informed consent forms to participate before participation. Patients were free to join or leave the study at any time. Additionally, all documents about patients' endoscopic findings and pathology results were codified and kept confidential.

Bacterial Isolation
All biopsy specimens were first mixed by a vortex for 50 s, and then 150 µl of homogenized suspension was streaked on Brucella agar plates enriched with 10% fetal bovine serum, 8% defibrinated caprine blood, and antibiotics (Selectab, MAST, UK). Plates were incubated at 37°C for 7–12 days under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2).[21,22] After incubation, translucent, grey and small colonies grew and confirmed by biochemical tests (for oxidase, urease, and catalase) to positively identify *H. pylori*. Moreover, microscopy was used to confirm the bacterial identity by observing typical characteristics, including curved rods and Gram-negative staining.[23,24] After confirming the identification, typical colonies were harvested and stored at −80°C in brain heart infusion growth medium containing 20% glycerol until further analysis.[25]

Antimicrobial Susceptibility Tests
In this assay, clarithromycin-susceptibility profile of confirmed *H. pylori* stocks was determined by using the E-test (AB Biodisk, Sweden). First, we have checked that the new plates did not have any contamination and then we placed an E-test strip on the surface of a plate.[23] Following 5–7 days of incubation at microaerophilic conditions at 37°C and with 95% humidity, we rechecked plates to determine zones of inhibited growth. The minimum inhibitory concentration (MIC) for clarithromycin was determined by the E-test following the manufacturer's instructions but with minor modifications. Strains were considered resistant when MIC was >0.05 µg/mL for clarithromycin.[23] Randomly, 10% of all isolates were selected for double-checking the first reported findings by a second operator.

DNA Extraction And Genotyping

Typical *H. pylori* colonies were carefully harvested, and DNA was extracted using a commercial kit according to the manufacturer's instructions (CinnaGen, Tehran, Iran). Extracted DNA was stored at −20°C for further analysis. *glmM* was used for genetic confirmation of *H. pylori* besides previous biochemical tests.[26] To determine the genotype of the resistant or susceptible strains, we used previously reported primers with minor changes.[27] Table 1 shows the primer sets used for PCR; distilled water was used as the negative control replacing the DNA template. To determine the positive or negative results, we subsequently subjected the PCR products (at least 4 μl) to electrophoresis on 1% agarose gels (Sina-clon, Tehran, Iran), and visualized the PCR products by a UV transilluminator (Biometra, Germany).

Statistical Analyses

A chi-square test was used to analyze the possible association between resistance status and particular genotypes (*cagA* and *vacA*). All measurements of significance were two-tailed with P < 0.05.

Results

Twenty-eight men and 33 women, with a mean age of 56.8 years (range, 21–73) were included. No statistically significant differences were found between age and gender of patients with *H. pylori* antibiotic resistance ($P < 0.05$). In our study, clarithromycin resistance rate was 21% (n = 13/61). Sixty-one out of 81 patients with duodenal ulcer, gastric ulcer and gastritis were recognized to be infected with *H. pylori* strains. Indeed, we discard the 20 samples due to lack of enough data from patients, or their disapproval to participate. Nevertheless, positive routine bacterial cultures, classic biochemical tests, and PCR amplification of *glmM* were used to identify the 61 positive cases. Generally, PCR for *glmM* detected 61 out of the 61 (100%) confirmed culture samples, indicating reliability and consistency of the two approaches.

Distribution Of *H. pylori cagA* And *vacA*

In total, 65% (n = 40/61) of the *H. pylori* strains were *cagA*-positive. The prevalence of *vacA* alleles were s_1/m_1 at 40% (n = 25/61), s_1/m_2 at 21% (n = 13/61), s_2/m_1 at 14% (n = 9/61), s_2/m_2 at %22 (n = 14/61). No statistical association was found between *vacA* alleles and gastroduodenal diseases ($P > 0.05$). The current genotyping on all the 61 *H. pylori* strains demonstrated that $vacAS_1M_1$ (the most virulent genotype based on *vacA*) was the predominant genotype in our study with no significant statistical discrepancy observed among the different disease diagnoses ($P > 0.05$). Distribution of the combined *cagA* and *vacA* genotypes of *H. pylori* among different diagnoses are summarized in Table 2.

Combination Of Susceptibility Profile With *H. pylori cagA-vacA* Genotypes

As shown in Table 2, clarithromycin-susceptible *H. pylori* isolates carried the $cagA^+$ $vacAs_1m_1^+$ genotype; this association was statistically significant ($P < 0.05$). The *H. pylori* $cagA^-$ $vacAs_1m_2^-$ genotype, as the less virulent strain, did not associate with the resistance phenotype ($P > 0.05$).

Discussion

Reportedly, *H. pylori* has colonized 50% of the world population.[28] The capacity of *H. pylori* to cause successful infections is affected by certain elements, which include carrying virulence factors, emergence of antibiotics resistance, and genetic vulnerability in the host.[29] Despite identification of *H. pylori* as the causative agent of various gastritis, designing the best standard therapy to eradicate

Table 1 Primer Sequences And Associated Amplification Conditions

Primer	Sequences	PCR Products	Amplification Conditions
glmM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294 bp	95 °C, 50 sec; 56 °C, 50 sec; 72 °C, 1 min (38 cycles)
cagA	ATAATGCTAAATTAGACAACTTGAGCGA TTAGAATAATCAACAAACATCACGCCAT	298 bp	95 °C, 55 sec; 58 °C, 50 sec; 72 °C, 1 min (36 cycles)
vacA s1/s2	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	259/286 bp	95 °C, 55 sec; 53 °C, 54 sec; 72 °C, 50 sec (37 cycles)
vacA m1/m2	CAATCTGTCCAATCAAGCGAG GCGTCAAAATAATTCCAAGG	567/642 bp	95 °C, 55 sec; 54 °C, 50 sec; 72 °C, 1 min (35 cycles)

Table 2 The $cagA^+vacAs_1m_1^+$ Was The Most Prevalent Genotype Among The Clarithromycin-Susceptible *H. pylori* Isolates

Resistance Status		Different Genotypes Among The Diseases Groups			
		$cagA\ vacAs_1m_1$	$cagA\ vacAs_1m_2$	$cagA\ vacAs_2m_1$	$cagA\ vacAs_2m_2$
Patients= (61)	**CLR-Susceptible** 13 (21%)	n = 12 (92%)	n = 1 (8%)	n = 0	n = 0
		DU: 6	DU:1	DU: 0	DU: 0
		GU: 4	GU: 0	GU: 0	GU: 0
		G: 2	G: 0	G: 0	G: 0
	CLR-Resistant 48 (89%)	n = 13 (27%)	n = 12 (25%)	n = 9 (19%)	n =14 (29%)
		DU: 9	DU: 5	DU: 5	DU: 5
		GU: 3	GU: 4	GU: 2	GU: 5
		G: 1	G: 3	G: 2	G: 4
P value		< 0.05	>0.05	>0.05	>0.05

Abbreviations: DU, duodenal ulcer, GU, gastric ulcer, G, gastritis, CLR, clarithromycin.

this bacterium is still in infancy stages.[30] From a biological point of view, the antibiotics-resistant *H. pylori* strains may have a higher chance to survive and propagate in human gastric microniches; hence, expecting certain virulence types among those pathogenic strains is plausible.[31,32] The main rationale for this research was based on this phenomenon that humans may harbor some *H. pylori* strains that carry virulence elements while they are also resistant to antibiotics. Among the Iranian population, *H. pylori* clarithromycin resistance is almost as high as 21%, which agrees with other parallel analyses.[33–35] However, a fundamental discord exists regarding the positivity of *cagA* and *vacA* alleles among the *H. pylori* strains. Our investigation for the first time has shown that clarithromycin-susceptible *H. pylori* strains carried the most virulent profile ($cagA^+vacAs_1m_1^+$). Importantly, some studies, such as that by Alarcón-Millán et al conducted in Mexico, reported no association between any virulence genotypes and clarithromycin-susceptibility patterns.[36] However, this major difference can be explained by geographical differences between Iran and Mexico. Moreover, we have found that 65% of the *H. pylori* strains are *cagA*-positive. The *H. pylori cagA*-positivity rate agrees with the rates reported in other studies, at least in Iran.[37–40] Little is known about any association between this virulence factor and clarithromycin susceptibility. Here, we showed, for the first time, that all most-virulent *H. pylori* strains are not clarithromycin-resistant, a report which should be seriously considered by gastroenterologists for better insight into treating this persistent microorganism. A multicenter study, including patients from different geographical regions, should be conducted to confirm our findings.

The main aim now is to eradicate this bacterium from the stomachs of symptomatic individuals; however, little is known about symptomatic patients likely infected by strains with antibiotics-susceptible profile. Such virulent strains (e.g., *cagA* and *vacA*) are more capable to induce acute inflammation with increased chance of causation of severe gastroduodenal diseases. In other words, we expect that *H. pylori* isolates representing the resistance genotypes have a higher chance to survive (according to the natural selection phenomena); thus, basically, they should have a quite different profile of virulence in comparison with antibiotics-susceptible isolates. Our study's limitations include: 1) relatively small number of subjects for antibiotic resistance analysis and 2) short period of time to investigate the clarithromycin susceptibility among *H. pylori* isolates. Indeed, we had only 1 year to analyze the subjects and assess the two virulent genotypes among both susceptible and resistant *H. pylori* isolates. Another basic limitation of our study is that we have not checked other virulence factors that may be associated with resistance. However, Alarcón-Millán et al used $babA_2$ as another possible virulence factor, but they have found no statistically significant association between resistance and *cagA* genotypes. At last, we were unable to check treatment failure among the patients who utilized clarithromycin within different therapeutic regimens. Indeed, more studies with larger sample sizes could facilitate thorough investigation of the relationship between virulent strains and susceptible *H. pylori* strains.

Conclusion

We found that some clarithromycin-susceptible *H. pylori* strains carry the virulent genotype, $cagA^+vacAs_1m_1^+$, and this can exacerbate the management of this persistent microorganism. Cumulative data indicate that both clarithromycin-resistant and clarithromycin-susceptible isolates of *H. pylori* need to be screened and tracked in regional populations. Thus, even the clarithromycin-susceptible *H. pylori* isolates should not be underestimated in the clinics.

Ethics

Our study was conducted in accordance with the Declaration of Helsinki.

Acknowledgments

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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