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Investigation of Clarithromycin Resistance-Associated Mutations and Virulence Genotypes of *Helicobacter pylori* Isolated from Iranian Population: A Cross-Sectional Study

Helia Alavifard[1] · Nasrin Mirzaei[1] · Abbas Yadegar[1] · Kaveh Baghaei[2] · Sinéad Marian Smith[3] · Amir Sadeghi[2] · Mohammad Reza Zali[2]

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Abstract

Antibiotic resistance has brought into question the efficiency of clarithromycin which is a vital component of eradication therapy for *Helicobacter pylori* infection. The point mutations within the 23S rRNA sequence of *H. pylori* isolates which contribute to clarithromycin resistance have yet to be fully characterized. This study was aimed to detect clarithromycin resistance-associated mutations and assess the prevalence of key virulence factors of *H. pylori* among Iranian patients. Amplification of 16S rRNA and *glmM* genes were done to identify *H. pylori*. Minimal inhibitory concentration (MIC) of clarithromycin in 82 *H. pylori* clinical isolates was determined by agar dilution method. Subsequently, various virulence markers including *cagA*, *vacA*, *sabA*, *babA*, and *dupA* of *H. pylori* were identified by PCR. PCR-sequencing was applied to detect point mutations in the 23S rRNA gene. Based on MIC values, 43.9% of *H. pylori* isolates showed resistance to clarithromycin. The *babA* and *cagA* genes were detected in 92.7% and 82.9% of isolates, assigned to be higher than other virulence factors. No significant relationship was found between the *H. pylori* virulence genotypes and clarithromycin susceptibility ($P > 0.05$). Analyzing the 23S rRNA sequences revealed A2143G (4/48, 8.3%) and A2142G (3/48, 6.2%) as the most prevalent mutations in clarithromycin-resistant isolates. Additionally, several novel mutations including G2220T, C2248T, A2624C, G2287A, T2188C, G2710C, C2248T, G2269A, and G2224T were also detected among either resistant or susceptible isolates. Our findings revealed the presence of several point mutations in the 23S rRNA gene of *H. pylori* isolates which may be associated with resistance to clarithromycin.

Introduction

Helicobacter pylori (*H. pylori*) has been recognized as a Gram-negative, microaerophilic microorganism which affects a large number of people on a global scale, with an increasing trend in developing countries. Since the role of *H. pylori* as the most successful human pathogen in the progression of chronic gastritis, peptic ulcer, gastric cell carcinoma and gastric mucosa-associated lymphoid tissue (MALT) was discovered, research on *H. pylori* eradication therapy began [1–3]. Several lines of empirical evidence revealed the reduction of gastric cancer owing to eradication of *H. pylori* infection [4]. However, the efficiency of *H. pylori* infection treatment has drastically dwindled due to factors such as antibiotic resistance, poor adherence to the eradication regimen, drug dosage administered, and duration of therapy. Of these, antibiotic resistance plays the most important role in *H. pylori* treatment failure [5]. Investigating antibiotic resistance from the molecular perspective

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✉ Abbas Yadegar
a.yadegar@sbmu.ac.ir; babak_y1983@yahoo.com

[1] Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[2] Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[3] School of Medicine & School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin 2, Ireland

highlights the notion that point mutations in the genome of *H. pylori* remain a big reason behind this global public health challenge [6]. Clarithromycin is an acid-stable and the most common bacteriostatic antibiotic which belongs to the macrolide group. This agent is frequently used as a vital component of standard triple therapy in first-line eradication regimens for *H. pylori* infection. Though, the efficiency of clarithromycin has been brought into question mostly due to antibiotic resistance [4]. It is often argued that the existence of cross-resistance in each family of antibiotics is due to the same resistance mechanism. Hence, resistance to clarithromycin implies resistance to all macrolides [7]. It is conceived that three-point mutations in the region of domain V of 23S rRNA, including A2142G, A2142C, and A2143G, mediate > 80% of *H. pylori* clarithromycin resistance in the world and ~ 90% of resistance in developed nations [8, 9]. However, the occurrence of point mutation at other positions such as A2115G, G2141A, T2117C, T2182C, T2717C, A2144G, C2147G, T2190C, C2195T, A2223G, and C2694A should not be overlooked [10, 11]. In the light of Maastricht V/Florence Consensus report, it is rational to perform susceptibility testing prior to first-line eradication therapy in areas with high clarithromycin resistance rates, since the prevalence of *H. pylori* antibiotic resistance varies in one region of a country over time and significantly from country to country. Furthermore, prescription of triple therapy containing clarithromycin should be averted when the primary clarithromycin resistance rate is more than 15–20% [4, 12–14].

A growing body of literature has investigated the correlation of antibiotic resistance with bacterial genetic factors, since an increasing trend in clarithromycin resistance has become a global concern [10]. A series of recent studies has indicated that bacterial virulence factors are categorized into three major pathogenic groups based on their ability to promote the pathogenesis of *H. pylori* infection. The groups are as follows: colonization (*babA*, *sabA*), immune escape (*cagA*, *vacA*), and disease induction (*cagA*, *vacA*, *babA*, *dupA*) [15, 16]. There are controversial arguments over the role of these virulence factors and antibiotic susceptibility. While recent finding reported a significant association between clarithromycin resistance and *vacA* i1 genotype; this view has been refuted by others [17, 18]. As might be expected, genotyping of *H. pylori* strains opens a new window for understanding a better picture of clinical outcomes, post-treatment follow-up and human migration [19]. Herein, we aimed to assess *H. pylori* antibiotic susceptibility rate in response to clarithromycin among Iranian patients with various gastroduodenal diseases. Additionally, we reported *H. pylori* 23S rRNA point mutations linked to clarithromycin resistance among Iranian clinical isolates. The prevalence of *H. pylori* virulence factors including *cagA*, *vacA*, *sabA*, *babA*, and *dupA* was also investigated.

Materials and Methods

Gastric Biopsy Samples

Antral biopsy specimens were collected from 82 previously determined *H. pylori*-infected patients, who underwent upper gastroduodenal endoscopy at Taleghani Hospital in Tehran from June 2017 to October 2019. The *H. pylori* infection in these patients was confirmed through culture, histological observations and RUT test. The biopsy samples were kept in transport medium consisting of thioglycolate supplemented with 1.3 g/L agar (Merck, Germany) and 3% yeast extract (Oxoid) and immediately were sent to the *Helicobacter* research laboratory. The exclusion criteria were those patients who took eradication therapy for *H. pylori* including PPIs, or H2-receptor blockers, and any antibiotics used for other infections within two weeks prior to enrollment. The study protocol was approved by the Institutional Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases at Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1395.878). All participants and/or their legal guardians signed written informed consent before enrollment in the study.

H. pylori Culture and Identification

The gastric biopsy specimens were completely homogenized and smeared on Brucella agar (Merck, Darmstadt, Germany) containing 7% horse blood (v/v), 10% fetal calf serum (FCS), and *Campylobacter*-selective supplement (vancomycin 2.0 mg/L, polymyxin B 0.05 mg/L, trimethoprim 1.0 mg/L). To prevent further fungal contamination, amphotericin B (2.5 mg/l) was added to the medium. The plates were incubated at 37 °C under microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) in a CO_2 incubator (Innova CO-170; New Brunswick Scientific, USA) for 3–7 days. Bacterial colonies were identified as *H. pylori* on the basis of phenotypic and biochemical tests including positive reactions for urease, catalase, and oxidase, and confirmed by PCR (16S rRNA and *glmM*) as previously described [20, 21].

Antimicrobial Susceptibility Testing

The antibiotic susceptibility of the *H. pylori* strains was determined by the agar dilution method against clarithromycin purchased from Sigma-Aldrich (St. Louis, MO, USA). Briefly, *H. pylori* suspensions were prepared in sterile saline and adjusted to a density equal to McFarland standard No. 3. The bacterial inocula were inoculated directly onto Mueller–Hinton agar (Merck, Darmstadt, Germany) plates

supplemented with 10% defibrinated horse blood containing clarithromycin dilutions ranging from 0.06 to 64 mg/L, and were incubated under microaerophilic conditions, as over-mentioned. The minimal inhibition concentration (MIC) was defined as the lowest concentration of antibiotic that thoroughly prevented the growth of the bacteria after 72 h of incubation. Clarithromycin MICs were interpreted based on the last guideline of European Committee on Antimicrobial Susceptibility Testing (EUCAST version 9.0, http://www.eucast.org/) [21]. Strains were considered to be susceptible for MIC of ≤ 0.25 mg/L, intermediate for MIC of 0.5 mg/L, and resistant for MIC of > 0.5 mg/L. A clarithromycin-susceptible *H. pylori* clinical isolate (strain HC114, NCBI/GenBank: MH040927.1) with previously identified MIC value served as a quality control strain [20].

Genotyping of *H. pylori* Virulence Determinants

DNA was extracted from single colonies from subcultures of each *H. pylori* strain using the QIAamp DNA extraction kit (QIAgen®, Hilden, Germany) according to the manufacturer's instructions. The concentration and integrity of DNA samples were verified by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The extracted DNA samples were stored at − 20 °C until further processing. PCR was used for virulence genotyping of *H. pylori* isolates. These virulence factors were as follows: *cagA*, *vacA* allelic types (s1/s2 and m1/m2), *babA2*, *sabA*, and *dupA* genes. The oligonucleotide sequences are presented in Table 1. PCR was performed in a final volume of 25 µl reaction mixture comprised of 2.5 µl 1X PCR buffer, 1 pmol of primers, 2 µl of DNA template (approximately 200 ng), 100 mM of dNTPs, 2 mM of $MgCl_2$, and 1.5 U/µL SuperTaq™ DNA polymerase (HT Biotechnology Ltd., Cambridge, UK). PCR amplifications were performed based on the previously described cycling programs [20, 22].

Primer Design for Detection of 23S rRNA Point Mutations

The NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) and the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) were used to access available complete 23S rRNA sequences of *H. pylori* strains. Based on sequence alignments of the 23S rRNA gene from various *H. pylori* strains and using the complete 23S rRNA sequence of *H. pylori* UA802 (U27270.1), specific primers for the conserved regions which cover all putative mutations associated with clarithromycin resistance were designed using CLC Sequence Viewer 8 (https://www.qiagenbioinformatics.com/), and Gene Runner Version 3.05 software. The expected size of the PCR amplicon was 1041 bp using primers 23S-F 5′-AGCACCGTAAGTTCGCGATAAG-3′ and 23S-R 5′-CTTTCAGCAGTTATCACATCC-3′. The primer specificity was checked in silico by the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis.

Direct Sequencing of 23S rRNA Gene

To detect specific mutations in the 23S rRNA gene of *H. pylori* strains, the direct PCR-based Sanger sequencing method was used for a selection of 50 susceptible and resistant strains. Briefly, PCR amplification was carried out in a final volume of 25 µl as over-mentioned under the cycling conditions: initial denaturation 94 °C for 4 min, followed by

Table 1 Oligonucleotide sequences used for molecular detection of *H. pylori* and virulence genotyping

PCR product (bp)	Annealing temperature (℃)	Oligonucleotide sequence (5′–3′)	Target gene
764	58	F: GGCTATGACGGGTATCCGGC R: GCCGTGCAGCACCTGTTTTC	16S rRNA
296	56	F: GGATAAGCTTTTAGGGGTGTTAGGGG R: GCTTACTTTCTAACACTAACGCGC	*glmM*
497	52	F: AACAGGACAAGTAGCTAGCC R: TATTAATGCGTGTGTGGCTG	*cagA*
259/286	57	F: ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	*vacA* s1/s2
570/645	57	F: CAATCTGTCCAATCAAGCGAG R: GCGTCAAAATAATTCCAAGG	*vacA* m1/m2
271	52	F: CCAAACGAAACAAAAAGCGT R: GCTTGTGTAAAAGCCGTCGT	*babA2*
487	56	F: TTTTTGTCAGCTACGCGTTC R: ACCGAAGTGATAACGGCTTG	*sabA*
488	55	F: ATTCACGCCTAAGACCTCA R: CTGAGAAGCCTTATTATCTTGTTGG	*dupA*

F forward, *R* reverse

30 cycles of 94 °C for 1 min, 56 °C for 45 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 10 min. The PCR amplicons were purified using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific, Fermentas, USA). Subsequently, both strands were sequenced by an automated sequencer (Macrogen, Seoul, Korea). DNA sequences were edited by Chromas Lite version 2.5.1 (Technelysium Pty Ltd, Australia) and BioEdit version 7.2.5 [23], and were aligned to the 23S rRNA sequence of *H. pylori* UA802 (U27270.1) as a reference sequence.

Phylogenetic Analysis

The edited 23S rRNA sequences of the *H. pylori* strains were aligned against reference sequence using ClustalW multiple alignment. A Maximum Likelihood tree was constructed by the Tamura 3-parameter model using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) [24]. Bootstrap values were based on 1000 replicates.

Statistical Analysis

Statistical association between *H. pylori* resistance to clarithromycin and virulence genotypes was analyzed by the non-parametric Chi-square and Fisher's exact tests using SPSS statistics version 23.0 for windows (IBM Corporation, Armonk, New York, USA). A P value of <0.05 was regarded statistically significant.

Results

Patient Characteristics

The *H. pylori*-infected patients consisted of 52 (45.6%) men and 30 (54.4%) women, with an average age of 49.7 ± 3.33 years old (range 20–80 years). Regarding endoscopic and pathological findings, 52 patients had non-ulcer dyspepsia (NUD), 18 had peptic ulcer disease (PUD), 11 had intestinal metaplasia (IM), and 1 had gastric cancer (GC). Table 2 summarizes the demographic characteristics of the patients in different clinical diagnosis groups.

Table 2 Demographic characteristics of *H. pylori*-infected patients in different clinical diagnosis groups

Parameter	Clinical diagnosis ($n=82$)			
	NUD ($n=52$)	PUD ($n=18$)	IM ($n=11$)	GC ($n=1$)
No. of isolates (%)	52 (63.4)	18 (21.9)	11 (13.4)	1 (1.2)
Mean age ± SD (year)	50.9 ± 4.39	44.4 ± 2.09	40.7 ± 3.51	63
Age range (year)	40–80	40–60	20–60	60–70
Gender (%)				
Female	16 (30.7)	8 (44.4)	6 (54.5)	0
Male	36 (69.2)	10 (55.5)	5 (54.5)	1 (100)

NUD non-ulcer dyspepsia, *PUD* peptic ulcer disease, *IM* intestinal metaplasia, *GC* gastric cancer

Clarithromycin Resistance of *H. pylori* Strains

The bacterial DNA samples were further used as a template for PCR reaction to identify *H. pylori* by amplification of 16S rRNA and *glmM* genes (Table 1). Accordingly, the clarithromycin resistance in 82 *H. pylori* strains was evaluated and their MIC values were recorded using the agar dilution method. By examining the MIC values, it can be inferred that 36 (43.9%) *H. pylori* strains were resistant to clarithromycin, whereas 42 (51.2%) strains showed susceptibility to this antimicrobial agent. Four (4.9%) strains were designated as intermediate ones. The MIC value of 16 mg/L was found as the most frequent breakpoint observed among the resistant strains, followed by MIC values of 1, 2, and 8 mg/L. The MIC values of 0.25 mg/L and 16 mg/L were determined as the MIC_{50} and MIC_{90} values, respectively. The frequency and distribution of MIC values for clarithromycin among *H. pylori* strains with respect to clinical diagnosis is presented in Table 3. The rate of resistance to clarithromycin was higher for the strains isolated from patients with PUD than NUD and IM patients. Frequency and distribution of clarithromycin resistance among *H. pylori* isolates in relation to different clinical diagnosis groups are shown in Table 4.

Table 3 Distribution of MIC values for clarithromycin among *H. pylori* isolates in different clinical diagnosis groups

CLR MIC (mg/L)	No	Clinical diagnosis ($n=82$)			
		NUD ($n=52$)	PUD ($n=18$)	IM ($n=11$)	GC ($n=1$)
0.06	28	19 (36.5%)	5 (27.7%)	3 (27.2%)	1 (100%)
0.125	9	5 (9.6%)	2 (11.1%)	2 (18.1%)	0
0.25	5	3 (5.8%)	1 (5.5%)	1 (9.1%)	0
0.5	4	2 (3.8%)	1 (5.5%)	1 (9.1%)	0
1	7	4 (7.7%)	2 (11.1%)	1 (9.1%)	0
2	6	5 (9.7%)	1 (5.5%)	0	0
4	2	1 (1.9%)	1 (5.5%)	0	0
8	5	2 (3.8%)	2 (11.1%)	1 (9.1%)	0
16	16	11 (21.1%)	3 (16.6%)	2 (18.1%)	0
32	0	0	0	0	0
64	0	0	0	0	0

CLR clarithromycin, *MIC* minimum inhibitory concentration, *NUD* non-ulcer dyspepsia, *PUD* peptic ulcer disease, *IM* intestinal metaplasia, *GC* gastric cancer

Table 4 Frequency and distribution of clarithromycin resistance and virulence genotypes among *H. pylori* isolates ($n=82$) in different clinical diagnosis groups

Parameter	Clinical diagnosis ($n=82$)				Total (%)
	NUD ($n=52$)	PUD ($n=18$)	IM ($n=11$)	GC ($n=1$)	
CLR resistance					
Susceptible	27 (51.9%)	8 (44.4%)	6 (54.5%)	1 (100%)	42 (51.2%)
Intermediate	2 (3.8%)	1 (5.5%)	1 (9.1%)	0	4 (4.9%)
Resistant	23 (44.2%)	9 (50%)	4 (36.3%)	0	36 (43.9%)
Virulence genotypes					
cagA-positive	39 (75%)	18 (100%)	10 (90.9%)	1 (100%)	68 (82.9%)
vacA alleles	22 (42.3%)	9 (50%)	4 (36.4%)	0	35 (42.7%)
s1m1	28 (53.8%)	8 (44.4%)	6 (54.5%)	1 (100%)	43 (52.4%)
s1m2	2 (3.8%)	1 (5.5%)	1 (9.1%)	0	4 (4.9%)
s2m2					
babA2-positive	47 (90.4%)	17 (94.4%)	11 (100%)	1 (100%)	76 (92.7%)
sabA-positive	35 (67.3%)	18 (100%)	10 (90.9%)	1 (100%)	64 (78%)
dupA-positive	31 (59.6%)	17 (94.4%)	9 (81.8%)	1 (100%)	58 (70.7%)

CLR clarithromycin, *NUD* non-ulcer dyspepsia, *PUD* peptic ulcer disease, *IM* intestinal metaplasia, *GC* gastric cancer

Association Between Virulence Genotypes and Clarithromycin Resistance

The resistant and susceptible clinical isolates were examined for the virulence factors of *H. pylori* including *cagA*, *dupA*, *babA*, *sabA*, and *vacA* (both allelic types: s and m regions). It can be seen clearly from the molecular results (Table 4) that the prevalence of *babA* and *cagA* genes were higher than others. In fact, these two genes were determined in 92.7% and 82.9% of the clinical isolates, respectively. When it comes to *vacA*, the prevalence of *vacA* s1m2 genotype was highest in the present study at 52.4%, while the least prevalent allelic combination was *vacA* s2m2 at 4.9%, and the *vacA* s1m1 combination was detected in 42.7% of the strains. *sabA*- and *dupA*-positive strains were found among 78% and 70.7% of the strains, respectively. Table 4 compares the data on the distribution of *H. pylori* genotypes among four groups of the patients. We failed to find a link between the prevalence of the virulence genotypes and clarithromycin susceptibility ($P>0.05$). In addition, the statistical analysis from this study found no significant correlation between clarithromycin susceptibility rate and different age and sex groups with various clinical diagnoses ($P>0.05$).

Sequence Analysis of 23S rRNA Gene Mutations

Regarding sequencing analysis in the present study, point mutations in the 23S rRNA gene were identified in 48 *H. pylori* isolates including 25 clarithromycin-resistant isolates, 22 clarithromycin-sensitive isolates, and one isolate with intermediate susceptibility. Moreover, the 23S rRNA gene was partially sequenced in two strains due to poor quality of sequence data or sequencing errors.

As shown in Supplementary Fig. 1, we observed 23 point mutations in the 23S rRNA gene sequence of the over-mentioned isolates. The list of point mutations in the 23S rRNA gene of *H. pylori* isolates is presented in Table 5. The type of mutations detected in the present study was not limited to A2142G and A2143G. Although the most prevalent mutations in clarithromycin-resistant isolates were A2143G (4/48, 8.3%) and A2142G (3/48, 6.2%), other point mutations including G2220T, T2221C, A2624C, C2248T, C2195T, T2182C, and C2288T were also detected with lower prevalence. In addition, some other single point mutations including G2287A, T2188C, G2710C, T2182C, C2248T, G2269A, G2224T, and C2694A were detected in clarithromycin-susceptible isolates. Apart from point mutations in the 23S rRNA gene, a transition T2244C and insertion of C nucleotide in position 2345 were observed in all clinical isolates including susceptible and resistant strains. Among the aforementioned point mutations, G2220T, C2248T, A2624C, G2287A, T2188C, G2710C, C2248T, G2269A, and G2224T were detected as novel point mutations.

Phylogenetic Analysis of 23S rRNA Gene

As might have been expected, the 23S rRNA gene is a highly conserved structure and no specific clustering in the phylogenetic tree was seen as a result. Figure 1 shows a clear illustration of similarities and discrepancies of 23S rRNA gene in different isolates based on the nucleotide sequence. However, strains with point mutations including A2142G and A2143G are classified in two separate clusters and the third branch implied to other point mutations in 23S rRNA gene sequence. The nucleic acid sequence of the 23S rRNA gene

Table 5 List of point mutations in the 23S rRNA gene of *H. pylori* isolates ($n=48$) observed in this study

Strains	GenBank Accession No.[a]	Clinical diagnosis	MIC (mg/L)	CLR susceptibility	Mutations
OC4	MH588172	PUD	16	Resistant	A2142G G2220T T2221C
OC15	MH588173	NUD	16	Resistant	None[a]
OC34	MH588174	PUD	0.06	Susceptible	None
HC70	MH588175	NUD	16	Resistant	A2142G
HC78	MH588176	NUD	0.25	Susceptible	G2287A
OC80	MH588177	NUD	0.06	Susceptible	None
OC81	MH588178	NUD	1	Resistant	A2624C
OC98	MH588179	NUD	8	Resistant	C2248T
HC133	MH588180	NUD	8	Resistant	None
HC136	MH588181	NUD	8	Resistant	A2142G
HC138	MH588182	PUD	16	Resistant	None
HC139	MH588183	NUD	0.06	Susceptible	None
OC149	MH588184	NUD	0.06	Susceptible	T2188C
OC152	MH588185	NUD	0.25	Susceptible	None
HC160	MH588186	NUD	16	Resistant	None
HC168	MH588187	PUD	0.06	Susceptible	None
HC175	MH588188	NUD	0.25	Susceptible	G2710C
OC175	MH588189	NUD	0.06	Susceptible	None
OC179	MH588190	GC	0.06	Susceptible	T2182C C2248T
HC180	MH588191	NUD	0.06	Susceptible	None
OC180	MH588192	IM	16	Resistant	A2143G
HC206	MH588193	PUD	8	Resistant	None
OC217	MH588194	NUD	0.06	Susceptible	None
OC218	MH588195	NUD	0.25	Susceptible	None
OC220	MH588196	NUD	8	Resistant	None
OC235	MH588197	PUD	0.5	Intermediate	None
OC245	MH588198	IM	16	Resistant	A2143G
OC250	MH588199	PUD	0.25	Susceptible	None
OC254	MH588200	NUD	1	Resistant	None
OC256	MH588201	NUD	1	Resistant	None
OC258	MH588202	NUD	16	Resistant	A2143G
OC359	MH588203	NUD	16	Resistant	None
OC485	MH588204	NUD	2	Resistant	None
OC494	MH588205	NUD	0.06	Susceptible	None
OC557	MH588206	PUD	2	Resistant	C2195T
OC571	MH588207	NUD	0.06	Susceptible	G2269A
OC576	MH588208	NUD	0.125	Susceptible	None
OC688	MH588209	IM	0.125	Susceptible	G2224T
OC797	MH588210	IM	16	Resistant	None
OC803	MH588211	NUD	1	Resistant	T2182C
OC810	MH588212	NUD	2	Resistant	C2195T
OC824	MH588213	PUD	0.06	Susceptible	None
OC840	MH588214	IM	1	Resistant	A2143G
OC852	MH588215	IM	2	Resistant	C2288T
OC937	MH588216	NUD	0.125	Susceptible	None
OC939	MH588217	PUD	4	Resistant	None
OC975	MH588218	IM	0.125	Susceptible	C2694A
OC1031	MH588219	NUD	0.06	Susceptible	None

CLR clarithromycin, *NUD* non-ulcer dyspepsia, *PUD* peptic ulcer disease, *IM* intestinal metaplasia, *GC* gastric cancer

[a]None, no specific variation detected as compared with 23S rRNA gene from clarithromycin-sensitive *H. pylori* strain UA802 (U27270.1)

and the relevant point mutations were deposited with GenBank/NCBI database under accession numbers MH588172 to MH588219.

Discussion

Clarithromycin is the main component of the first-line treatment regimens for *H. pylori* infection [25]. However, World Health Organization (WHO, 2017) presented clarithromycin-resistant *H. pylori* in the category of high priority which demands thoughtful and profound attention on eradication regimens for this problematic infection [26, 27]. Highlighting the importance of clarithromycin-resistant *H. pylori*, we decided to study the prevalence and the main mechanisms involved in clarithromycin resistance among clinical isolates of *H. pylori*. Regarding the Bangkok Consensus Report, the clarithromycin resistance rate is considered high when it exceeds 10–15%. Based on this statement, it can distinguish geographical regions into high- and low-resistance areas [28]. Thus, it can be inferred that region studied in present study is a high-resistance area with 43.9% clarithromycin-resistant strains, which showed an upward trend to our previous investigation with 33.8% of *H. pylori* resistance to clarithromycin [20].

With respect to a recent meta-analysis by Savoldi et al. [4], the high prevalence of primary clarithromycin resistance was observed in the Western Pacific Region (34%) and Eastern Mediterranean Region (33%). Regarding clarithromycin-resistant *H. pylori* in Europe, it was estimated to be more than 15%, whereas the lowest prevalence of clarithromycin resistance was assessed for Americas Region and the South-East Asia Region with similar primary resistant pattern (10%).

Increasing evidence supports a rising concern about the global resistance rate of clarithromycin among *H. pylori* strains. For instance, assessment of *H. pylori* primary antibiotic resistance in the Asia–Pacific region was performed by Kuo et al. [29]. This study revealed that the overall prevalence of resistance to clarithromycin was 17%, and total antibiotic resistance soared from 7% before 2000 to 21% in 2011–2015. With regards to studies from Iran, the overall prevalence of clarithromycin-resistant *H. pylori* strains has been reported to be 14.7%. The lowest and the highest resistance rates belonged to Rasht and Kashan with 5.5% to 33.7%, respectively [30]. A similar conclusion was reached by Hakemi Vala et al. [31] who reported that 21.7% of the *H. pylori* isolates were resistant to clarithromycin, whilst our findings in Tehran, the same city, revealed a notably higher resistance rate (43.9%). These findings extend our knowledge that clarithromycin resistance has been increasing during a period and as a consequence, the efficacy of clarithromycin-based treatment will be dramatically decreased. In the face of such an increasing trend, the third Maastricht consensus conference drew attention to the fact that the eradication rate of *H. pylori* should be more than 80% for an intention-to-treat analysis if a promising treatment is considered [25]. With reference to Hellenic consensus, treatment regimens for *H. pylori* should be based on regional antibiotic resistance rates and individual consumption of antibiotics [32].

When it comes to the point mutations responsible for clarithromycin resistance, frequent mutations in the gene encoding 23S rRNA have been discussed by a great number of authors in the literature. There is also accumulating evidence that single point mutations including A2143G (69.8%), A2142G (11.7%), and A2142C (2.6%) are the most common alterations seen in 23S rRNA sequence [32, 33], which is in line with our findings. In our study, seven isolates were found to harbor A2142G and A2143G mutations, whilst no A2142C mutation was seen among the resistant isolates. Furthermore, we identified a number of other mutations including G2220T, T2221C, A2624C, C2248T, C2195T, T2182C, and C2288T in addition to the most frequent ones. Matta et al. [33] reported the mutations A2144G, T2183C, and C2196T in clinical practice for the first time in Colombia, whereas none of those mutations were observed in the present study. Versalovic et al. [34] observed that 91.5% of clarithromycin-resistant isolates of *H. pylori* carried either the A2143G (formerly A2058G) or the A2144G (formerly A2059G) mutations in the gene encoding 23S rRNA. According to their findings, the majority of A2143G mutations were present in isolates with MICs more than 64 mg/L. On the contrary, the highest MIC in the present study was 16 mg/L. A similar pattern of results was obtained by Keshavarz Azizi Raftar et al. [35] who found A2143G with the prevalence of 66.7% among clarithromycin-resistant *H. pylori*. However, the high existence of A2143G may increase the concerns regarding treatment failure. In agreement with these studies, those sequences with mutations A2143G had the highest MIC (16 mg/L) among our clarithromycin-resistant strains. An interesting finding of our study was that several sequences of clarithromycin-resistant *H. pylori* were devoid of any mutations, indicating other potential mechanisms such as efflux pumps may be involved in clarithromycin resistance [36]. To the best of our knowledge, single point mutations including G2220T, A2624C, G2287A, T2188C, G2710C, C2248T, G2269A, and G2224T are considered as putative point mutations involved in clarithromycin resistance. Thus, further studies are required to assess the role of these mutations in the development of clarithromycin resistance among *H. pylori* strains.

Regarding the global prevalence of *H. pylori* and antibiotic resistance, a growing amount of resources are being devoted to analyses of virulence factors and antibiotic resistance between *H. pylori* strains. One of these main virulence

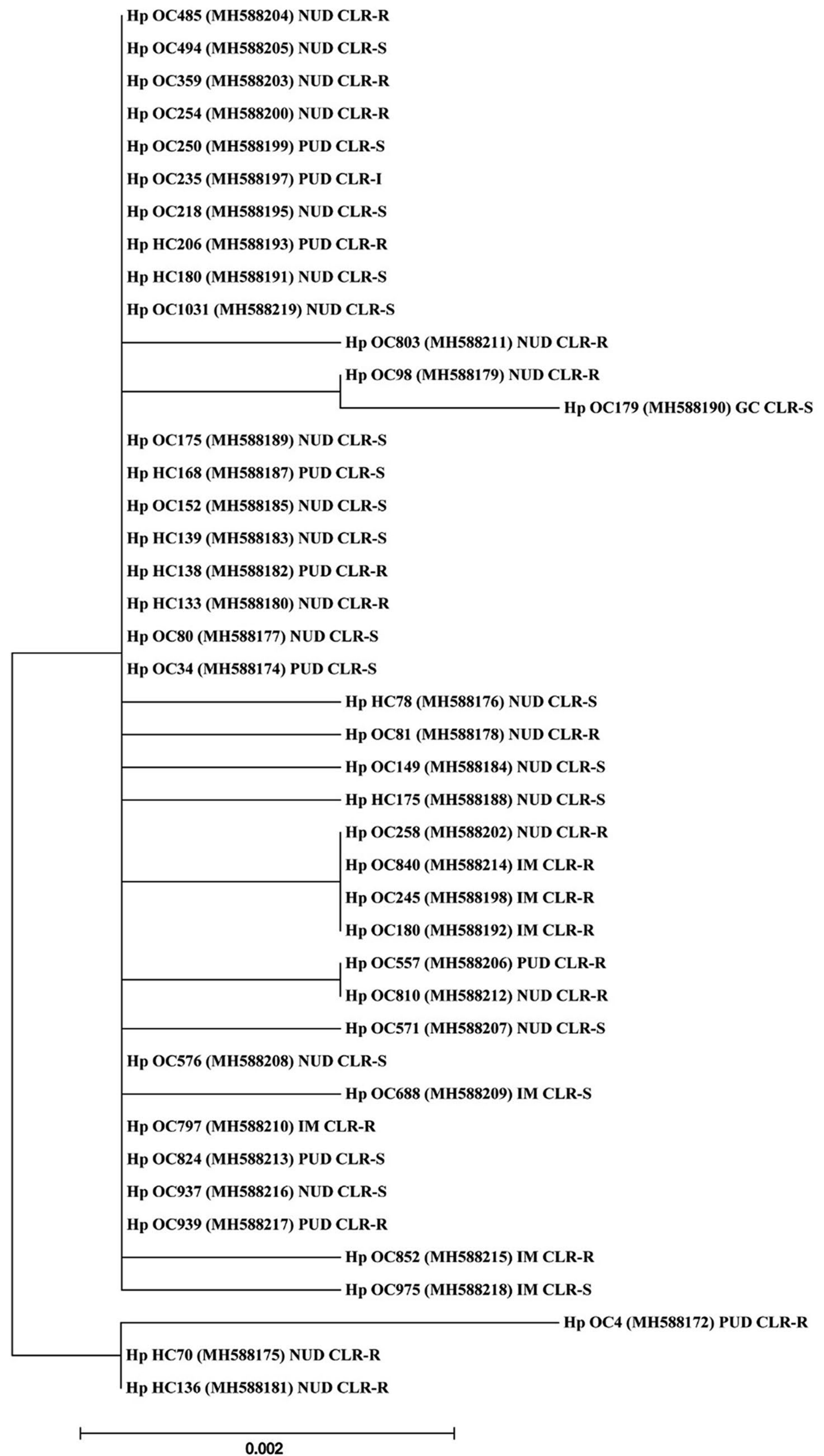

Fig. 1 Phylogenetic tree of *H. pylori* clinical strains based on 23S rRNA nucleotide sequences ($n=43$). Maximum likelihood tree of concatenated sequences was constructed using MEGA7 software with bootstrap method at 1000 replications. The evolutionary distances were computed using the Tamura 3-parameter model

factors is *vacA,* which carries two variable regions including the signal sequence (s) and middle region (m). The *vacA* s1 genotype is associated with PUD and the most virulent type of *vacA* s1m1 is related to gastric carcinoma, whereas inactive toxin is produced by the s2m2 allelic type [37]. Other findings provide evidence for the prevalence of *vacA* among *H. pylori* isolates as allelic combinations s1m1, s2m2, and s1m2 or s2m1 in 57.7%, 33.3%, and 4.4%, respectively [38]. It is interesting to note that the existence of s1m1 is more probable in patients with peptic ulcer in Western countries [39]. Conversely, in our study the allelic combination s1m2 had the highest prevalence and it was more common in those patients who suffered from NUD. Furthermore, *H. pylori* strains which are *cagA*-genopositive and carry *vacA* s1m1 concurrently, increase the risk of gastric precancerous lesions by roughly 4.8-fold in infected patients in comparison with those who were infected with *cagA*-negative/*vacA* s2m2 strains [15]. Although no significant correlation was found between antibiotic susceptibility and virulence factors in the present study, there exists a considerable body of literature on association of primary clarithromycin resistance with less virulent strains of *H. pylori* (*cagA*-negative and *vacA* s2) [40]. Additionally, Elviss et al. [41] argued that clarithromycin susceptibility in *H. pylori* was related with *vacA* s1m2 genotype. Intriguingly, Khani et al. [42] reported clarithromycin-susceptible *H. pylori* strains harbored the most virulent profile ($cagA^{+}$/*vacA* $s1m1^{+}$).

In a recent study by Farzi et al. [20], the *vacA* s1m2 genotype was the most common allelic type which was found to be correlated with PUD. Their results also showed that other *vacA* alleles including s1m1 and s2m2 genotypes were observed in 27.9% and 26.5% of the isolates, respectively. As might be reasonably expected, the concurrent expression of multiple virulence genes can escalate the risk of a severe gastric disorder [43]. Indeed, the decrease of *babA* expression can be contributed to higher incidence of gastrointestinal damages [3]. With reference to *dupA*, findings have shown a clear-cut positive correlation between the presence of *dupA* gene with increased risk of duodenal ulcer [15]. It follows the fact that clarithromycin resistance rate was ordinarily lower in the *dupA*-positive in contrast to *dupA*-negative group [44]. As for *sabA*, the frequency of this gene in *H. pylori* strains was 100%, 86.7%, and 83.3% in patients with gastric cancer, gastric ulcer, and both gastritis and duodenal ulcer, respectively [45]. There is still considerable controversy surrounding the association between *H. pylori* strains and clinical outcome. The recent data firmly established that no meaningful association was perceived among pathogenicity and antibiotic resistance or susceptibility which is completely in agreement with the present study [18, 46]. Despite the present study has successfully determined the prevalence of *H. pylori* virulence factors as well as molecular detection of clarithromycin resistance-associated mutations, it has some important limitations. First, a limited number of *H. pylori* 23S rRNA sequences were sequenced in this work. Thus, further investigations using a large cohort of isolates are necessary to determine additional mutations which are responsible for resistance to clarithromycin by themselves or in combination. Also, there could be other possible mechanisms involved in clarithromycin resistance that were not evaluated in the present study.

Conclusion

In summary, the prevalence of clarithromycin-resistant *H. pylori* isolates has been reaching a worrying level in Iran. Our findings revealed the presence of several point mutations in the 23S rRNA gene of *H. pylori* clinical isolates which may be associated with acquisition of resistance to clarithromycin. Additionally, the present data showed a high prevalence of *H. pylori* virulence factors among Iranian patients. Taken together, antimicrobial surveillance should be performed frequently to monitor the current susceptibility patterns and its alterations in each geographical region. Accordingly, it provides a strong empirical evidence for clinicians to replace clarithromycin or apply a potential combination of antibiotics to get the best results. Though, from the epidemiological point of view, it is important to be aware of the distribution of virulence factors in *H. pylori* isolates. Further studies using local surveillance networks are required to choose appropriate *H. pylori* eradication regimens for each region.

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Author Contributions HA performed the susceptibility testing and PCR assay for *H. pylori* strains. NM provided the draft of the manuscript. AY worked on concept and design of the study, data analysis and interpretation, and writing of manuscript. AY and KB performed the sequence analysis. AY and SMS critically revised the manuscript. AS and MRZ provided clinical consultation for the study. All authors approved the final version of the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to declare.

Ethical Approval The study protocol was approved by the Institutional Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases at Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1395.878).

Consent to Participate All participants and/or their legal guardians signed written informed consent before enrollment in the study.

Consent for Publication All authors approved the final version of the manuscript and the authorship list.

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