High prevalence of antibiotic resistance in Helicobacter pylori isolates from Iran: importance of functional and mutational analysis of resistance genes and virulence genotyping

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Abstract

The high prevalence of antibiotic resistance in *Helicobacter pylori* has become a great challenge in Iran. The genetic mutations that contribute to the resistance have yet to be precisely identified. This study aimed to investigate the prevalence of antibiotic resistance and virulence markers in Iranian *H. pylori* isolates and to analyze if there is any association between resistance and genotype. Antibiotic susceptibility patterns of 33 *H. pylori* isolates were investigated against metronidazole, clarithromycin, amoxicillin, rifampicin, ciprofloxacin, levofloxacin and tetracycline by the agar dilution method. The *frxA*, *rdxA*, *gyrA*, *gyrB* and 23S rRNA genes of the isolates were sequenced. The virulence genotypes were also determined using PCR. Metronidazole resistance was present in 81.8% of the isolates, followed by clarithromycin (36.4%), ciprofloxacin (36.4%), amoxicillin (30.3%), rifampicin (30.3%), levofloxacin (27.3%) and tetracycline (6.1%). Most of the metronidazole-resistant isolates carried frameshift mutations in both *frxA* and *rdxA* genes, and premature termination was occurred in positions Q5Stop and Q50Stop, respectively. Amino acid substitutions M191I, G208E, and V199A were predominantly found in *gyrA* gene of fluoroquinolone-resistant isolates. A2143G and C2195T mutations of 23S rRNA were found in four isolates. Interestingly, significant associations were demonstrated between intact *cag*PAI and resistance to rifampicin (*P* = 0.027), and between susceptibility to amoxicillin and *cag*PAI intactness (*P* = 0.016). The prevalence of *H. pylori* antibiotic resistance is high in our region, particularly that of metronidazole, clarithromycin, ciprofloxacin and multidrug resistance. Occurrence of mutations in resistance genes were involved in the development of resistance, especially in less virulent isolates.

**Keywords:** *Helicobacter pylori*; Resistance genes; Mutations; Virulence genotype, *cag*PAI intactness
**Introduction**

*Helicobacter pylori* (*H. pylori*) is known as the most common human pathogen infecting more than half of the world’s population.\(^1,2\) Early eradication based therapies have been proven to regress the *H. pylori*-associated diseases.\(^3,4\)

However, the efficacy of eradication treatments has been extremely compromised primarily due to increased resistance to antimicrobial agents in many countries.\(^5-8\)

Today, first-line standard triple therapy is the most widely used eradication treatment for *H. pylori* infection, which typically comprises two of three antibiotics including amoxicillin, clarithromycin, and metronidazole in combination with one proton pump inhibitor (PPI).\(^3,9\) However, the use of levofloxacin or ciprofloxacin in fluoroquinolone containing triple therapy and bismuth-based quadruple therapy have also been suggested as second-line therapies after the failure of the clarithromycin-containing regimens.\(^10-12\) Furthermore, tetracycline and rifampicin are among the common antibiotics that have been used in several rescue therapies recommended in eradication of *H. pylori* infection.\(^13-15\)

Previous studies have demonstrated that numerous point mutations resulting from genetic plasticity within the chromosomal genes, is the main antibiotic resistance mechanism among *H. pylori* strains in various geographic regions.\(^5,6,16-18\) Primary resistance to clarithromycin has been mainly associated with point mutations in the peptidyl transferase region encoded in domain V of 23S rRNA. Most of these mutations include nucleotide substitutions involving an adenine to guanine transition at positions 2142 and 2143, and to a lesser extent an adenine to cytosine transversion at position 2142.\(^8,10,19\) However, several other mutations associated with clarithromycin resistant isolates seem to be emerging.\(^20,21\) The mechanisms of metronidazole resistance in *H. pylori* are frequently attributed to inactivating mutations in *rdxA* and *frxA* genes.\(^22,23\) On the other hand, mutational changes leading to various amino acid substitutions that confer fluoroquinolone resistance have been located in different positions of quinolone-resistant determining region (QRDR) of *gyrA* and *gyrB* genes.\(^19,24\)

Apart from aforementioned mechanisms of resistance developed by *H. pylori* strains to the major antibiotics used in the treatment of infection, other factors such as the virulence genotype status of bacteria have been reported to affect drug resistance.\(^25-28\) However, the exact underlying mechanisms involved in the crosstalk of *H. pylori* virulence and antimicrobial resistance remained to be clarified.

Hence, the focus of the present study was to evaluate the antibiotic susceptibility patterns and underlying resistance mechanisms of *H. pylori* strains isolated from Iranian patients with different gastric diseases. Furthermore, we
determined the presence of genetic mutations that are associated with antibiotic resistance. We also examined the possible association between resistance profiles and a panel of virulence genotypes.

**Methods**

**Patients and *H. pylori* isolates**

Antral biopsies were collected for culture from 78 patients who underwent upper gastroduodenal endoscopy at Taleghani Hospital in Tehran from February 2016 to September 2016. Patients were excluded if they were taking eradication therapy for *H. pylori*, PPIs or H2-receptor blockers, and any antibiotics used for other infections within two weeks prior to enrolment. The study protocol was approved by the 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 experiments were performed in accordance with relevant guidelines and regulations recommended by the institution and informed consents were obtained from all subjects and/or their legal guardians prior to sample collection.

The biopsy specimens were smeared on Brucella agar (Merck, Germany) plates containing 7% horse blood (v/v), 10% fetal calf serum (FCS), Campylobacter-selective supplement and amphotericin B (2.5 mg/l). The inoculated plates were incubated at 37°C in a CO2 incubator under microaerophilic atmosphere containing approximately 5% O2, 10% CO2 and 85% N2 for 3-10 days. The *H. pylori* was identified by colony and microscopic morphology, positive catalase, oxidase, and urease tests and confirmed by molecular assays.29,30

**Antibiotic susceptibility testing**

The antibiotic susceptibility of the *H. pylori* strains was assessed by the agar dilution method against a panel of 7 antibiotics purchased from Sigma-Aldrich (St. Louis, MO, USA), including metronidazole (MNZ), clarithromycin (CLR), amoxicillin (AMX), rifampicin (RIF), ciprofloxacin (CIP), levofloxacin (LEV), and tetracycline (TCN). The range of antibiotic concentrations was as follows: 0.25-256 mg/L for MNZ, 0.06 to 64 mg/L for CLR, 0.03 to 4 mg/L for AMX, 0.03 to 32 mg/L for RIF and LEV, 0.06 to 32 mg/L for CIP, and 0.06 to 16 mg/L for TCN. *H. pylori* inoculums were prepared from 72 h-old cultures that were suspended in sterile saline and adjusted to a density equal to No. 3 McFarland standard. The bacterial suspensions were inoculated directly onto Mueller-Hinton blood agar (Merck, Germany) plates supplemented with 10% defibrinated horse blood containing antibiotic dilutions, and were incubated under microaerophilic conditions as over-mentioned. After 72 hours of incubation, the minimal inhibition concentrations (MICs) were determined as the lowest concentration of antibiotic that completely inhibited the growth.
of the inoculums. The resistance breakpoints were used as described by the last guideline of European Committee on Antimicrobial Susceptibility Testing (EUCAST). Strains were considered to be resistant for MICs of >8 mg/L for MNZ, >0.125 mg/L for AMX, and >1 mg/L for RIF, CIP, LEV, and TCN. Clarithromycin MICs were interpreted based on CLSI breakpoints (≤0.25 mg/L, susceptible; 0.5 mg/L; intermediate; ≥1.0 mg/L, resistant). A clinical isolate of H. pylori with previously identified MIC values was served as a quality control strain in all susceptibility tests.

Genomic DNA extraction

Subcultures of the single colonies were prepared, and confluent cultures from each colony were used for DNA extraction using QIAamp DNA extraction kit (QIAgen®, Hilden, Germany) following the manufacturer’s directions. The DNA samples were stored at -20 °C until used for gene amplification.

Mutation analysis of the resistance genes

To detect specific mutations in the frxA, rdxA, gyrA, gyrB and 23S rRNA genes, a PCR-based sequencing approach was carried out in all H. pylori isolates including the susceptible and resistant strains. The frxA and rdxA genes were amplified as described by Han et al. Amplification of gyrA and gyrB genes were performed using primers as described elsewhere. Mutations within bacterial 23S rRNA peptidyl transferase gene were assessed as presented by Ho et al. The oligonucleotide primers are shown in Table 1. The PCR products were sequenced on both strands using an automated sequencer (Macrogen, Seoul, Korea). All complete and partial DNA sequences were edited by Chromas Lite version 2.5.1 (Technelysium Pty Ltd, Australia). Comparative sequence analysis between resistant and sensitive strains was carried out using BioEdit software version 7.2.5. The DNA and deduced amino acid sequences were aligned and coordinated to H. pylori 26695 (GenBank: CP003904.1) as a reference sequence.

Detection of virulence markers

The presence of virulence factors including cagA, cagL, vacA alleles (s1/s2 and m1/m2), babA2 and sabA genes were assessed based on our previously published work. The diversity of cagA C-terminal variable region and intactness of cagPAI was analysed as previously described. A PCR-sequencing assay was also used to analyse the functional (on/off) status of oipA gene. To investigate the presence of dupA gene, we used the previously designed primers by Jung et al. H. pylori J99 (CCUG 47164) and a no-template reaction were used as positive and negative controls in all amplifications, respectively.

Nucleotide sequence accession numbers
The sequences obtained from this study were submitted to NCBI under the following GenBank accession numbers:

- Domain V 23S rRNA, MH040926-MH040949
- gyrA, MH054262-MH054292
- gyrB, MH054293-MH054319
- frxA, MH054320-MH054346
- rdxA, MH054347-MH054374

**Statistical analysis**

The SPSS Statistics for Windows (version 21.0, Armonk, NY: IBM Corp.) was used to perform all statistical analyses. The Chi-square and Fisher’s exact tests were used to determine the statistical significance of differences between categorical variables. A $P$ value of less than 0.05 was considered as statistically significant.

**Results**

**Characteristics of patients**

Totally, 33 (42.3%) $H.\text{ pylori}$ isolates were cultured from antral biopsies of the patients included in the study. The $H.\text{ pylori}$ infected patients consisted of 14 (42.4%) men and 19 (57.6%) women, with an average age of $49.7 \pm 9.7$ years old (range 28-75 years). Endoscopic diagnosis showed that 15 patients had chronic gastritis (CG), 12 had peptic ulcer disease (PUD), and 6 had intestinal metaplasia (IM).

**Prevalence of antibiotic resistance**

Overall, metronidazole resistance was the highest (27/33, 81.8%), and the lowest resistance rate was observed against tetracycline in 2/33 (6.1%) isolates. Resistance to clarithromycin, amoxicillin and rifampicin was observed in 12/33 (36.4%), 10/33 (30.3%), and 10/33 (30.3%) of isolates, respectively. Three (9.1%) isolates were found as intermediate to clarithromycin. $H.\text{ pylori}$ resistance to ciprofloxacin and levofloxacin was detected in 12/33 (36.4%), and 9/33 (27.3%) of isolates, respectively. Only one isolate was found to be susceptible to all antibiotics examined. The rate of resistance to metronidazole, clarithromycin, amoxicillin and levofloxacin was higher in patients with PUD and IM than with CG patients. Inversely, the rate of resistance to rifampicin was higher in CG patients than with PUD and IM. There were no important differences in the rate of resistance to ciprofloxacin and tetracycline between CG and with PUD and IM patients. All patients with IM were resistant to metronidazole. The MIC range, MIC50/MIC90, prevalence of resistance and distribution of MIC values for the $H.\text{ pylori}$ strains are shown in Tables 2 and 3.

**Multi-drug resistance**

Single-drug resistance (SDR) was observed in 9 (27.3%) isolates, in which resistance to metronidazole was the most frequent SDR phenotype (5/9, 55.5%). Totally, 23/33 (69.7%) isolates showed multidrug resistance (MDR) phenotype, and 16 different MDR profiles were detected. No isolate was resistant to all tested antibiotics.
distribution of the SDR and MDR profiles within various clinical outcome groups is shown in Table 4. Resistance to
MNZ + AMX and MNZ + RIF were equally the most common double-drug resistance profiles (2/6, 33.3%). Resistance to MNZ + CLR + CIP was found as the most frequent triple-drug resistance profile (3/11, 27.3%). All of the isolates from patients with IM and more than half of the PUD isolates showed MDR phenotype, mostly having triple-drug resistance profile.

**Genetic variations of frxA and rdxA genes**

Totally, 27 of the frxA and 28 of the rdxA genes obtained from all isolates were sequenced and analysed as shown in Table 5. Fourteen (51.8%) isolates exhibiting resistance to metronidazole predominantly carried insertions and/or deletions resulting in translational frameshift mutations in the FrxA. One isolate was found to have stop codon at position Q5Stop resulting in premature termination codon (PTC), while missense mutations were found in 11/27 (40.7%) isolates. In addition, about one-third (10/28, 35.7%) of the isolates were found to have frameshift mutations in rdxA gene. Nonsense mutations resulting in PTC were identified in 3/28 (10.7%) isolates due to codon substitutions at position Q50Stop of RdxA. Missense mutations were distributed among 5 susceptible and 10 resistant isolates of the rdxA genes. One resistant strain had no mutation in both genes. The peptide sequence alignments for frxA and rdxA genes from metronidazole-susceptible and -resistant isolates in comparison with reference strain are presented in Supplementary Figs S1 and S2, respectively.

**Amino acid variations at QRDR region of gyrA and gyrB genes**

As shown in Table 6 and Supplementary Figs S3 and S4, selective regions in the QRDR of gyrA and gyrB genes were sequenced among 31 and 27 H. pylori isolates, respectively. Totally, 16 different amino acid substitutions were detected in gyrA subunit among all isolates. Three different amino acid variants including S63P, R140K, and A183V were detected to be exclusively present in gyrA of the fluoroquinolone-resistant isolates, whereas six different substitutions of A97V, D143E, A207T, G208K, I212S, and E214K were found to be present in the susceptible isolates only. In addition, seven other mutations were observed at D86N, D86G, V150A, M191I, V199A, G208A, and G208E in both fluoroquinolone-susceptible and -resistant isolates. The most frequent substitutions in gyrA of the fluoroquinolone-resistant isolates were M191I (14/23, 60.9%), G208E (13/25, 52%), and V199A (5/9, 55.5%), respectively. The M191I-G208E substitution was found as the most common double mutations (8/12, 66.7%) within 5 resistant and 3 susceptible isolates, while the M191I-V199A-G208E was found as the most frequent triple
substitutions (3/9, 33.3%) from 2 susceptible and one resistant isolates. The quadruple substitution was detected in

\textit{gyrA} of 3 resistant and 3 susceptible isolates.

As for \textit{gyrB} subunit, two different amino acid variants including D481E and R484K were detected among 7 isolates. The D481E substitution was found to be present in both fluoroquinolone-susceptible and -resistant isolates, whereas R484K was exclusively present in resistant isolates. As shown in Table 6 and Supplementary Fig S4, two fluoroquinolone-susceptible isolates had the single D481E mutations, while 5 resistant isolates presented the double D481E-R484K only. No mutation of \textit{gyrB} was found in 11 fluoroquinolone-resistant and 9 susceptible isolates.

**Genetic variations of 23S rRNA gene**

The domain V of 23S rRNA gene was sequenced in 24 \textit{H. pylori} isolates. As shown in Supplementary Fig S5, this region was highly conserved with minimal nucleotide variations in comparison to \textit{H. pylori} strain 26695 as the reference genome. Overall, four nucleotide transitions including A2143G and C2195T were identified in clarithromycin-resistant isolates. None of these mutations were observed among the susceptible isolates and no isolates were found to have double mutations of A2143G and C2195T. The distribution of MIC values according to the different mutations in all phenotypically resistant and susceptible isolates is presented in Table 7.

**Association between virulence genotypes and resistance patterns**

The frequency and distribution of strains grouped by virulence genotypes according to each susceptibility pattern is shown in Table 8. High frequencies of \textit{cagL}-positive and \textit{cagA}-positive genotypes were found frequently in susceptible isolates for all antibiotics tested, with the exception of metronidazole. The \textit{cagA} ABC motif was also found frequently in susceptible isolates, with the exception of metronidazole and clarithromycin. \textit{H. pylori} isolates with \textit{vacA} s1m2 were found more frequently in susceptible isolates. Strains with \textit{oipA} “on” status and \textit{babA}, \textit{sabA} and \textit{dupA} positivity were also frequently found in susceptible isolates. There were only two isolates that showed resistance against tetracycline and both these strains also were \textit{sabA}-positive. All ciprofloxacin-resistant isolates also were found to be \textit{dupA}-positive. There was no association between these virulence factors and antibiotic resistance patterns ($P > 0.05$). Furthermore, \textit{H. pylori} strains harboring intact or partial \textit{cagPAI} were variably distributed between susceptible and resistant isolates. Interestingly, significant associations were observed between intact \textit{cagPAI} and resistance to rifampicin ($P = 0.027$), and between susceptibility to amoxicillin and \textit{cagPAI} intactness ($P = 0.016$).

**Discussion**
Eradication of *H. pylori* infection has been reported to significantly improve the clinical outcome of infected patients in high-risk areas.\(^3,4\) However, the eradication rate of *H. pylori* has been decreasing progressively, mainly due to increased resistance to antimicrobial agents, especially in developing countries.\(^5,6,11,17,41-43\) Thus, in order to choose the appropriate antibiotics in different *H. pylori* treatment regimens, we need to have recently updated susceptibility data in the local setting. In Iran, nearly 40-90\% of the adult population is infected with *H. pylori*, which seems to be acquired early in childhood.\(^32,37,44\) There have been few reports on the antibiotic resistance of *H. pylori* in Iran by performing agar dilution method as the reference method for this bacterium.\(^32,45-47\) However, none of the previous studies investigated the functional and molecular mechanisms that contribute to resistance of *H. pylori* strains from Iranian patients. Therefore, we carried out this work to determine the molecular characteristics of genes involved in antibiotic resistance, and evaluate the association between resistance patterns and a wide panel of virulence genotypes.

The prevalence of metronidazole resistance was reported to be high among Iranian *H. pylori* strains and ranged from 40.5\% to 78.6\%.\(^45,47,48\) The results of this study showed an increased rate of metronidazole resistance (81.8\%) as compared to previous reports from Iran.\(^32,45,47\) A very high prevalence of metronidazole resistance has also been reported from other developing countries in Asia including Bangladesh (77.5\%), China (95.4\%), India (83.8\%), Kuwait (70\%), Pakistan (89\%), and Vietnam (69.9\%).\(^43,49-53\) The extremely high rate of metronidazole resistance observed in this study might be attributed to widespread and unauthorized consumption of antimicrobial drugs in Iran.

In addition, massive use of metronidazole in the treatment of various infections such as anaerobic bacterial and parasitic infections, and for diarrheal, dental, periodontal, and gynecologic diseases could explain the significantly high rate of metronidazole resistance in in many developing countries.\(^5,32,41,43\) Therefore, in agreement with other previous studies *H. pylori* treatment regimens containing metronidazole are not useful and should not be chosen as first-line eradication therapy in Iran.\(^5,41-43,54\)

Previous studies demonstrated that various point mutations in *frxA* and *rdxA* genes were linked to metronidazole resistance in *H. pylori*.\(^23,51,55\) As expected, different types of mutations including insertions, deletions, missense, nonsense and frameshift mutations were detected among the studied strains. Our results showed that most of the metronidazole-resistant isolates presented frameshift mutations in these genes. Moreover, we found point mutations introducing stop codon at positions Q5Stop and Q50Stop in *frxA* and *rdxA* genes, respectively. Many other nonsense mutations that lead to PTC have also been reported in *rdxA* and rarely in *frxA* genes.\(^5,17,22,33,41,56,57\) However, in this study one of the metronidazole-resistant isolate did not contain any alterations in both *frxA* and *rdxA* genes. As
previously suggested, metronidazole resistance in this small subset of isolates may be due to the presence of additional resistance mechanisms and mutations in other redox enzymes. \(^{33,41,58}\)

Fluoroquinolones were proven to have bacteriostatic activities by trapping DNA gyrase and topoisomerase IV. These drugs considered as salvage treatment for *H. pylori* eradication in second- or third-line therapies after the failure of clarithromycin-based treatment regimens. \(^{12,24,59}\) However, it has been reported that fluoroquinolone resistance is rapidly expanding around the world. \(^{6,7,53}\) In a previous study from Iran, the rate of resistance to ciprofloxacin and levofloxacin was reported about 27% and 24.3%, respectively. \(^{32}\) In this study, we found a significant increase of fluoroquinolone resistance, which is of great concern. Nevertheless, studies from Taiwanese and Malaysian populations revealed that gemifloxacin is superior to levofloxacin in antimicrobial activity and may have better drug efficacy than levofloxacin in *H. pylori* eradication. \(^{41,60}\)

Point mutations in the QRDR of *gyrA* and *gyrB* sequences, greatly reduce the antimicrobial activity of fluoroquinolones. To date, several mutations have been identified in *gyrA* subunit of *H. pylori* strains from different geographical regions. \(^{5,8,34,41,60-64}\) None of the most common mutations in *gyrA* hot spot positions N87K, D91N, and D91G were detected in our isolates. However, 10 novel substitutions including S63P, D143E, A183V, A207T, G208K, G208A, G208E, I212S, E214K, and M191I were identified in the *gyrA* of either/both fluoroquinolone-resistant or/susceptible strains in this study. Among them mutations M191I, G208E, and V199A were predominantly found in fluoroquinolone-resistant isolates. Moreover, *gyrB* mutations may rarely occur and have little impact on primary fluoroquinolone resistance. \(^{5,8,34,41,61,64}\) In this study, only two amino acid changes D481E and R484K were identified in *gyrB*, in which R484K was exclusively present in resistant strains.

Among macrolides, clarithromycin is recognized as a major antibiotic for *H. pylori* eradication therapy because of its impact on treatment outcomes. \(^{20,65}\) The rate of clarithromycin resistance is topically much lower than that to metronidazole. However, the rate of primary clarithromycin resistance is undoubtedly on the rise and varies between different geographical regions. \(^{7,8,43,53,66}\) Unfortunately, the level of clarithromycin resistance in this study increased in comparison to a previous study \(^{32}\) from 26 to 36.4%, which is of great concern.

It has been claimed that three most frequently reported mutations, including A2143G, A2142G and A2142C, are responsible for more than 90% cases of primary resistance to clarithromycin. \(^{67,68}\) However, in a recent study by De Francesco *et al.*, this concordance was reduced to only 54.8%, with the A2142C mutation not being detected at all. \(^{20}\)

Moreover, some other mutations have been found to be associated with clarithromycin resistance, although their
precise role is not yet clear. In this study, only four mutations including A2143G and C2195T were found in our isolates. We failed to identify additional mutations such as T2183C and A2223G, which are frequently reported to be the cause of clarithromycin resistance in Eastern countries, rather than in Western countries. Additionally, no point mutation was identified in the sequence of 23S rRNA gene in four clarithromycin-resistant strains. For those isolates, we can speculate that other resistance mechanisms, such as the presence of an efflux pump, may be implicated in development of resistance to clarithromycin.

It is estimated that the overall resistance rates to amoxicillin and tetracycline are 23.61% and 7.38% in Asian countries, respectively. Similarly, we observed high rate of resistance to these drugs among the studied isolates (30.3% to amoxicillin and 6.1% to tetracycline), which is a matter of great concern in H. pylori eradication in Iran. However, the level of resistance to these antibiotics reported to be very low or even absent in most western countries versus African countries. Regarding rifampicin, we also observed a rising rate of resistance from 14.4% to 30.3% in comparison to a previous report. Recently, Regnath et al. reported considerable increase in resistance to rifampicin from 3.9% to 18.8% between 2002 and 2015 among pediatric patients from southwest Germany.

Unfortunately, emergence of MDR H. pylori strains has become a serious challenge all over the world. In a previous study, the resistance rate to at least two antimicrobial agents was reported in 43% of the H. pylori isolates from Iran. Surprisingly, our finding showed that 69.7% of the isolates were resistant to at least two antibiotics. The high prevalence of MDR phenotype may be attributed to the exhaustive use of antibiotics across the country. Information about the prevalence of quadruple-drug resistance is limited, and a few reports from India (2.5%), Bulgaria (0.7%), Vietnam (1.9%) and Indonesia (2.6%) are available yet. However, 18.7% of the isolates in this study showed quadruple-drug resistance, which was lower than the previous study (37.9%). Moreover, resistance to tetracycline was only observed in the isolates with quadruple-drug resistance. This finding could be explained to the presence of multidrug efflux pumps in these strains.

There have been several reports on the relationship between H. pylori virulence markers and antibiotic resistance. Accordingly, patients infected with cagA-positive strains that also carry more virulent vacA alleles significantly have high cure rates and eradication success than less virulent strains. It has been hypothesized that colonization of gastric mucosa by more virulent H. pylori genotypes may induce a higher degree of inflammation and increase blood flow, which in turn can favor better diffusion of the antibiotics. Alternatively, another possible explanation may be related to the fact that cagA-positive strains proliferate faster than cagA-negative ones and would therefore be more
susceptible to antibiotics. Furthermore, Taneike et al. observed that cagA-negative strains may tend to acquire spontaneous drug resistance under selective pressure of antimicrobials. However, it still remains somewhat controversial because recent reports indicated that these virulent genotypes variously distributed between susceptible and resistant strains. CagA protein with a greater number of EPIYA-C repeats is considered to be pathophysiologically more virulent and carcinogenic. Thus, according to the over-mentioned hypothesis, we expected the presence of more virulent types of CagA EPIYA motifs in susceptible isolates than resistant ones. However, as the number of EPIYA types having two or more EPIYA-C repeats was very low, we could not come to such conclusion. Similar to other studies performed in Italy (37.2%) and North Wales (53%) and Germany (37.4%), vacA s1m2 (48.5%) genotype was the most prevalent vacA mosaicisms in our strains. Although H. pylori strains with vacA s1m2 were detected more frequently in metronidazole-resistant isolates, no significant associations was found. In contrast, vacA s1m2 genotype was found more frequently in susceptible isolates for other antibiotics examined. Likewise, isolates with oipA “on” status and harboring babA, sabA and dupA genotypes were frequently found in metronidazole-resistant isolates. On the other hand, all of these genotypes were frequently found in susceptible isolates for other antibiotics, with the exception of sabA in levofloxacin-resistant ones. Moreover, two isolates that showed resistance against tetracycline were sabA-positive, and all ciprofloxacin-resistant isolates were found to be dupA-positive. However, we found no association between these virulence factors and antibiotics resistance ($P > 0.05$). H. pylori strains that carry an intact and functional cagPAI are more virulent and frequently associated with severe clinical outcomes than those carrying partial or no cagPAI. As far as we know, this is the first study that relates the cagPAI integrity with antibiotic resistance. Our results showed that H. pylori isolates harboring intact or partial cagPAI were variably distributed between susceptible and resistant isolates. However, we found significant associations between intact cagPAI and resistance to rifampicin ($P = 0.027$), and contrastingly between susceptibility to amoxicillin and cagPAI intactness ($P = 0.016$). These results are contradictory and did not strongly support the idea that susceptibility to antibiotics is higher in infections caused by more virulent genotypes. Nevertheless, it is likely that infected patients with resistant and hypervirulent strains are at increased risk of progression to more severe clinical outcomes due to failure in H. pylori eradication.

In conclusion, this study demonstrated that the prevalence of H. pylori antibiotic resistance is worrisome in our country with rising trend over the time. The findings from this study also highlight the relevance of different types of mutations in genes responsible for antibiotic resistance in H. pylori strains. We also provide evidence for the importance of
simultaneous screening of the virulence and resistance genotypes in *H. pylori* strains for guiding clinicians to choose an appropriate combination of drugs. Taken together, because of alarming increase in the rate of *H. pylori* antibiotic resistance in our local population, it is reasonable to constantly monitor the antimicrobial susceptibility patterns, and develop effective treatment and preventive strategies at national level.

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**Author Contributions**

N. Farzi collected the *H. pylori* strains, performed the susceptibility testing and molecular assays. A. Yadegar worked on concept and design of the study, data analysis and interpretation, and writing of manuscript. A. Sadeghi, H. Asadzadeh Aghdaei, and M. R. Zali critically revised the paper. All authors approved the final version of the manuscript and the authorship list.

**Supplementary Information**

Supplementary information associated with this article can be found, in the online version.
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| Target gene | Primer designation | Oligonucleotide sequence (5’ - 3’) | Annealing temperature (°C) | PCR product (bp) | Reference |
|-------------|---------------------|-----------------------------------|----------------------------|-----------------|-----------|
| frxA        | frx1                | TGGATATGGCAGCCGTTTA               | 52                         | 729             | Han 2007  |
|             | frx2                | GGTTATCAAAAAAGCTAACAGCG          |                            |                 |           |
| rdxA        | rdx1                | ATGGTAATTGTTCGTTAGGG             | 48                         | 758             | Han 2007  |
|             | rdx2                | CTCCTGAACTTTAATTTAG              |                            |                 |           |
| gyrA        | gyrAPF              | AGCTTAATCCATGAGCGTGGA            | 52                         | 582             | Wang 2010 |
|             | gyrAPR              | TCAGGCCCTTTGACAAATTCC           |                            |                 |           |
| gyrB        | gyrBPF              | CCCTAAGCAAGCCAAAAATCA            | 51                         | 465             | Wang 2010 |
|             | gyrBPR              | GGGCGCAAAATAACGATAGAA           |                            |                 |           |
| 23S rRNA    | Hp23-1              | CCACAGCGATGTGGTCTCAG             | 54                         | 425             | Ho 2010   |
|             | Hp23-2              | CTCCATAAGAGCCAAAAAGCCC          |                            |                 |           |
Table 2. Distribution of the antibiotic resistance patterns, MIC range, MIC$_{50}$ and MIC$_{90}$ values for each antibiotic among *H. pylori* isolates used in this study.

| Antibiotic agents | MIC range | MIC$_{50}$ | MIC$_{90}$ | No. (%) of MIC (mg/L) |
|-------------------|-----------|-----------|-----------|-----------------------|
|                   |           |           |           | Susceptible | Resistance |
| MNZ               | 0.25-128  | 32        | 128       | 6 (18.2)    | 27 (81.8)  |
| CLR$^a$           | 0.06-16   | 1         | 16        | 18 (54.5)   | 12 (36.4)  |
| AMX               | 0.03-0.5  | 0.25      | 0.5       | 23 (69.7)   | 10 (30.3)  |
| CIP               | 0.06-32   | 2         | 16        | 21 (63.6)   | 12 (36.4)  |
| LEV               | 0.03-32   | 2         | 16        | 24 (72.7)   | 9 (27.3)   |
| RIF               | 0.03-32   | 2         | 16        | 23 (69.7)   | 10 (30.3)  |
| TCN               | 0.06-16   | 16        | >16       | 31 (93.9)   | 2 (6.1)    |

MNZ, metronidazole; CLR, clarithromycin; AMX, amoxicillin; CIP, ciprofloxacin; LEV, levofloxacin, RIF, rifampicin; TCN, tetracycline

$^a$Three (9.1%) *H. pylori* isolates had intermediate susceptibility against clarithromycin based on CLSI breakpoints (MIC values equal to 0.5 mg/L).
Table 3. Distribution of MIC values for each antibiotic among *H. pylori* isolates used in this study.

| MIC (mg/L) | MNZ | CLR | AMX | CIP | LEV | RIF | TCN |
|------------|-----|-----|-----|-----|-----|-----|-----|
| 0.03       |     | 11  | 3   | 3   | 3   | 2   | 3   |
| 0.06       | 10  | 30  | 10  | 1   | 2   | 4   | 2   |
| 0.12       | 6   | 18.2| 2   | 6.1 | 7   | 21.2| 5   |
| 0.25       | 3   | 9.1 | 2   | 6.1 | 5   | 15.2| 6   |
| 0.5        | 3   | 9.1 | 5   | 15.2| 3   | 9.1 | 5   |
| 1          | 2   | 6.1 | 3   | 9.1 | 4   | 12.1| 1   |
| 2          | 4   | 12.1| 4   | 12.1| 1   | 3   | 9   |
| 4          | 1   | 3   | 1   | 3   | 1   | 3   | 3   |
| 8          | 2   | 6.1 | 2   | 6.1 | 2   | 6.1 | 2   |
| 16         | 7   | 21.2| 4   | 12.1| 6   | 18.2| 2   |
| 32         | 6   | 18.2| 1   | 3   | 1   | 3   | 2   |
| 64         | 8   | 24.2| 2   | 6.1 | 2   | 6.1 | 2   |
| 128        | 5   | 15.2| 2   | 6.1 | 2   | 6.1 | 2   |
| 256        | 2   |     | 2   | 6.1 | 2   | 6.1 | 2   |

MNZ, metronidazole; CLR, clarithromycin; AMX, amoxicillin; CIP, ciprofloxacin; LEV, levofloxacin; RIF, rifampicin; TCN, tetracycline.
Table 4. Distribution of the multidrug resistance profiles in relation to clinical outcomes among *H. pylori* isolates used in this study.

| Resistance profiles | Clinical outcome | Total No. (%) |
|---------------------|------------------|---------------|
|                     | CG (n = 14)      | PUD (n = 12)  | IM (n = 6) |
| Single drugs        |                  |               |            |
| CLR                 | 0                | 1             | 0          | 1 (3.1) |
| AMX                 | 1                | 0             | 0          | 1 (3.1) |
| RIF                 | 1                | 0             | 0          | 1 (3.1) |
| CIP                 | 0                | 1             | 0          | 1 (3.1) |
| MNZ                 | 3                | 2             | 0          | 5 (15.6) |
| Double drugs        |                  |               |            |
| MNZ + AMX           | 1                | 1             | 0          | 2 (6.2) |
| MNZ + RIF           | 1                | 0             | 1          | 2 (6.2) |
| CIP + RIF           | 1                | 0             | 0          | 1 (3.1) |
| MNZ + CIP           | 0                | 1             | 0          | 1 (3.1) |
| Triple drugs        |                  |               |            |
| MNZ + CLR + LEV     | 0                | 1             | 1          | 2 (6.2) |
| MNZ + AMX + RIF     | 1                | 1             | 0          | 2 (6.2) |
| MNZ + CLR + CIP     | 1                | 1             | 1          | 3 (9.4) |
| MNZ + CLR + AMX     | 0                | 0             | 1          | 1 (3.1) |
| MNZ + AMX + CIP/LEV | 0                | 1             | 0          | 1 (3.1) |
| MNZ + RIF + CIP/LEV | 2                | 0             | 0          | 2 (6.2) |
| Quadruple drugs     |                  |               |            |
| MNZ + CLR + AMX + LEV | 0            | 1             | 0          | 1 (3.1) |
| MNZ + CLR + AMX + CIP/LEV | 0 | 0 | 1 | 1 (3.1) |
| MNZ + CLR + TCN + LEV | 1            | 0             | 0          | 1 (3.1) |
| MNZ + TCN + RIF + LEV | 0            | 0             | 1          | 1 (3.1) |
| MNZ + CLR + AMX + CIP | 0                | 1             | 0          | 1 (3.1) |
| MNZ + CLR + CIP + RIF | 1                | 0             | 0          | 1 (3.1) |

MNZ, metronidazole; CLR, clarithromycin; AMX, amoxicillin; CIP, ciprofloxacin; LEV, levofloxacin; RIF, rifampicin; TCN, tetracycline; CG, chronic gastritis; PUD, peptic ulcer disease; IM, intestinal metaplasia.
Table 5. Number of nucleotide insertion and deletion in \( frxA \) and \( rdxA \) genes involved in metronidazole resistance among \( H. pylori \) isolates used in this study.

| Strains | Resistance phenotype | MIC (mg/L) | No. of nucleotide ins/del \( frxA \) | Mutation | No. of nucleotide ins/del \( rdxA \) | Mutation | SDR/MDR | Clinical Outcome |
|---------|----------------------|------------|---------------------------------|----------|---------------------------------|----------|---------|-----------------|
| OC80    | Susceptible          | 0.25       | None                            | In-frame | None                            | In-frame | N\(^a\)  | CG              |
| OC112   | Resistant            | 32         | ND\(^b\)                        | In-frame | ND\(^b\)                        | In-frame | MDR     | CG              |
| HC114   | Resistant            | 16         | Ins (2)/Del (4)                 | Frameshift | None                            | In-frame | SDR     | PUD             |
| HC138   | Resistant            | 32         | Del (2)                         | Frameshift | None                            | In-frame | MDR     | PUD             |
| HC168   | Resistant            | 32         | Del (1)                         | Frameshift | ND\(^b\)                        | ND\(^b\) | MDR     | PUD             |
| OC179   | Resistant            | 64         | ND\(^b\)                        | In-frame | ND\(^b\)                        | ND\(^b\) | SDR     | CG              |
| OC180   | Resistant            | 32         | ND\(^b\)                        | Frameshift | Del (2)                         | Frameshift | MDR     | IM              |
| OC217   | Resistant            | 32         | Del (1)                         | Frameshift | Ins (1)                         | Frameshift | MDR     | IM              |
| OC218   | Susceptible          | 0.25       | ND\(^b\)                        | Frameshift | ND\(^b\)                        | ND\(^b\) | SDR     | CG              |
| OC235   | Resistant            | 32         | ND\(^b\)                        | Frameshift | Ins (9)                         | Frameshift | MDR     | PUD             |
| OC245   | Resistant            | 8          | ND\(^b\)                        | Frameshift | ND\(^b\)                        | ND\(^b\) | MDR     | IM              |
| OC250   | Susceptible          | 1          | None\(^a\)                      | In-frame | None                            | In-frame | SDR     | PUD             |
| OC485   | Resistant            | 128        | Del (1)                         | Frameshift | None\(^a\)                      | In-frame | MDR     | CG              |
| OC494   | Susceptible          | 0.25       | None\(^a\)                      | In-frame | None                            | In-frame | MDR     | CG              |
| OC557   | Resistant            | 64         | Del (3)                         | Frameshift | None                            | In-frame | MDR     | PUD             |
| OC562   | Susceptible          | 8          | None\(^a\)                      | In-frame | None                            | In-frame | SDR     | CG              |
| OC571   | Resistant            | 64         | None\(^a\)                      | In-frame | None                            | In-frame | MDR     | CG              |
| OC576   | Resistant            | 64         | Del (1)                         | Frameshift | Q50Stop                         | PTC      | SDR     | CG              |
| OC688   | Resistant            | 16         | Del (1)                         | Frameshift | Q50Stop                         | PTC      | MDR     | IM              |
| OC797   | Resistant            | 16         | Del (1)                         | Frameshift | None                            | In-frame | MDR     | IM              |
| OC803   | Resistant            | 16         | None\(^a\)                      | In-frame | None                            | In-frame | MDR     | CG              |
| OC810   | Resistant            | 64         | None\(^a\)                      | In-frame | Del (1)                         | Frameshift | MDR     | CG              |
| OC824   | Resistant            | 128        | None\(^a\)                      | In-frame | Q50Stop                         | PTC      | MDR     | PUD             |
| OC840   | Susceptible          | 1          | None\(^a\)                      | In-frame | None                            | In-frame | SDR     | PUD             |
| OC852   | Resistant            | 16         | ND\(^b\)                        | Ins (4)   | Frameshift                      | MDR     | IM     | PUD             |
| OC897   | Resistant            | 16         | None\(^a\)                      | In-frame | Ins (6)/Del (2)                 | Frameshift | SDR     | PUD             |
| OC912   | Resistant            | 16         | Del (1)                         | Frameshift | Del (1)                         | Frameshift | MDR     | PUD             |
| OC913   | Resistant            | 128        | Ins (3)/Del (1)                 | Frameshift | None                            | In-frame | MDR     | PUD             |
| OC937   | Resistant            | 128        | ND\(^b\)                        | Del (1)   | Frameshift                      | SDR     | CG     | PUD             |
| OC939   | Resistant            | 64         | Q5Stop                          | PTC       | None                            | In-frame | MDR     | PUD             |
| OC975   | Resistant            | 64         | None\(^a\)                      | In-frame | ND\(^b\)                        | ND\(^b\) | MDR     | IM              |
| OC985   | Resistant            | 64         | None\(^a\)                      | In-frame | Del (1)                         | Frameshift | MDR     | CG              |
| OC1031  | Resistant            | 128        | Ins (2)                         | Frameshift | Del (1)                         | Frameshift | MDR     | CG              |

Ins, nucleotide insertion; Del, nucleotide deletion; PTC, premature termination codon; SDR, single-drug resistance; MDR, multidrug resistance; CG, chronic gastritis; PUD, peptic ulcer disease; IM, intestinal metaplasia

\(^a\)None, no specific variation detected as compared with genes or amino acids from metronidazole-sensitive \( H. pylori \) isolates

\(^b\)ND, not determined (the obtained sequence was not appropriate for mutational analysis)

\(^c\)N, not resistant to all antibiotics tested
| Strains | Resistance phenotype | MIC (mg/L) CIP/LEV | Mutations | gyrA | gyrB | SDR/MDR | Clinical Outcome |
|---------|----------------------|--------------------|-----------|------|------|---------|-----------------|
|         |                      | CIP LEV            |           |      |      |         |                 |
| OC80    |Susceptible/Susceptible|0.5 0.06           |M191I, V199A, G208E |D481E |None |         | CG              |
| OC112   |Susceptible/Susceptible|0.25 0.25          |M191I, G208E |None |      |         | MDR CG          |
| HC114   |Susceptible/Susceptible|0.12 0.12          |M191I, G208E |None |      |         | SDR PUD         |
| HC138   |Resistant/Susceptible  |16 0.5             |V150A, M191I, G208E |None |      |         | MDR PUD         |
| HC168   |Susceptible/Susceptible|0.25 0.06          |D143E, G208K, I212S, E214K |ND |      |         | SDR CG          |
| OC179   |Susceptible/Susceptible|0.5 0.06           |M191I, V199A, G208A |ND |      |         | MDR CG          |
| OC180   |Susceptible/Susceptible|0.12 0.06          |V150A, M191I, V199A, G208E |None |      |         | MDR IM          |
| OC217   |Susceptible/Susceptible|0.12 0.06          |M191I, G208E |ND |      |         | MDR CG          |
| OC218   |Susceptible/Susceptible|0.12 0.06          |ND |      |ND |         | SDR CG          |
| OC235   |Susceptible/Susceptible|0.25 0.06          |D86N, M191I, G208E |None |      |         | MDR PUD         |
| OC245   |Resistant/Resistant   |16 16              |M191I, V199A, G208A |None |      |         | MDR IM          |
| OC250   |Resistant/Susceptible  |16 0.5             |D86G, M191I |None |      |         | SDR PUD         |
| OC485   |Resistant/Susceptible  |2 0.12             |D86N, A183V, M191I |None |      |         | MDR CG          |
| OC494   |Resistant/Susceptible  |32 0.5             |None |      |None |         | MDR CG          |
| OC557   |Susceptible/Resistant  |0.12 16            |V199A, G208E |None |      |         | MDR PUD         |
| OC562   |Susceptible/Susceptible|1 0.12             |D86G, M191I, A207T, G208E |ND |      |         | SDR CG          |
| OC571   |Resistant/Resistant   |16 16              |D86G, M191I, V199A, G208E |None |      |         | MDR CG          |
| OC576   |Susceptible/Susceptible|0.06 0.5           |V199A, G208E |D481E |      |         | SDR CG          |
| OC688   |Susceptible/Susceptible|1 1                |M191I, V199A, G208E |None |      |         | MDR IM          |
| OC797   |Resistant/Susceptible  |2 0.03             |M191I, V199A, G208E |D481E, R484K |      |         | MDR IM          |
| OC803   |Resistant/Susceptible  |2 0.03             |S63P, M191I, V199A, G208E |None |      |         | MDR CG          |
| OC810   |Susceptible/Resistant  |1 32               |M191I, G208E |None |      |         | MDR CG          |
| OC824   |Resistant/Resistant   |16 16              |M191I, G208E |D481E, R484K |      |         | MDR PUD         |
| OC840   |Susceptible/Susceptible|1 0.06             |G208E |None |      |         | SDR PUD         |
| OC852   |Susceptible/Resistant  |0.5 2              |M191I, G208E |D481E, R484K |      |         | MDR IM          |
| OC897   |Susceptible/Susceptible|0.25 0.03          |A97V, G208E |None |      |         | SDR PUD         |
| OC912   |Resistant/Resistant   |2 0.12             |G208E |D481E, R484K |      |         | MDR PUD         |
| OC913   |Susceptible/Resistant  |0.25 16             |M191I, G208E |D481E, R484K |      |         | MDR PUD         |
| OC937   |Susceptible/Resistant  |0.12 0.5            |G208E |None |      |         | SDR CG          |
| OC939   |Resistant/Resistant   |4 0.12             |M191I, G208E |None |      |         | MDR PUD         |
| OC975   |Susceptible/Resistant  |0.25 16             |D86N, R140K, M191I, G208E |None |      |         | MDR IM          |
| OC985   |Susceptible/Susceptible|0.12 0.06          |ND |      |None |         | MDR CG          |
| OC1031  |Resistant/Resistant   |16 16              |D86N, M191I, G208E |ND |      |         | MDR CG          |

Table 6. Mutations in *gyrA* and *gyrB* genes involved in fluoroquinolone resistance among *H. pylori* isolates used in this study.

CIP, ciprofloxacin; LEV, levofloxacin; SDR, single-drug resistance; MDR, multidrug resistance; CG, chronic gastritis; PUD, peptic ulcer disease; IM, intestinal metaplasia.

*None, no specific variation detected as compared with genes or amino acids from fluoroquinolone-sensitive *H. pylori* isolates.*

*ND, not determined (the obtained sequence was not appropriate for mutational analysis).*

*N, not resistant to all antibiotics tested.*

The *gyrA* quinolone-resistant determining regions of the strains OC250, OC485 and OC494 were partially translated due to low quality of obtained sequences.
Table 7. Mutations in 23S rRNA gene involved in clarithromycin resistance among *H. pylori* isolates used in this study.

| Strains | Resistance phenotype | MIC (mg/L) | Mutations | SDR/MDR | Clinical Outcome |
|---------|----------------------|------------|-----------|----------|-----------------|
| OC80    | Susceptible          | 0.062      | None*     | N°       | CG              |
| OC112   | Susceptible          | 0.125      | ND°       | MDR      | CG              |
| HC114   | Susceptible          | 0.062      | None*     | SDR      | PUD             |
| HC138   | Resistant            | 16         | None*     | MDR      | PUD             |
| HC168   | Susceptible          | 0.062      | None*     | MDR      | PUD             |
| OC179   | Susceptible          | 0.062      | ND°       | SDR      | CG              |
| OC180   | Resistant            | 16         | A2143G    | MDR      | IM              |
| OC217   | Susceptible          | 0.062      | ND°       | MDR      | CG              |
| OC218   | Susceptible          | 0.25       | ND°       | SDR      | CG              |
| OC235   | Intermediate         | 0.5        | None*     | MDR      | PUD             |
| OC245   | Resistant            | 16         | ND°       | MDR      | IM              |
| OC250   | Susceptible          | 0.25       | None*     | SDR      | PUD             |
| OC485   | Resistant            | 2          | None*     | MDR      | CG              |
| OC494   | Susceptible          | 0.062      | None*     | MDR      | CG              |
| OC557   | Resistant            | 2          | C2195T    | MDR      | PUD             |
| OC562   | Intermediate         | 0.5        | ND°       | SDR      | CG              |
| OC571   | Susceptible          | 0.25       | None*     | MDR      | CG              |
| OC576   | Susceptible          | 0.125      | ND°       | SDR      | CG              |
| OC688   | Susceptible          | 0.125      | None*     | MDR      | IM              |
| OC797   | Resistant            | 16         | None*     | MDR      | IM              |
| OC803   | Resistant            | 1          | ND°       | MDR      | CG              |
| OC810   | Resistant            | 2          | C2195T    | MDR      | CG              |
| OC824   | Susceptible          | 0.062      | None*     | MDR      | PUD             |
| OC840   | Resistant            | 1          | A2143G    | SDR      | PUD             |
| OC852   | Resistant            | 2          | ND°       | MDR      | IM              |
| OC897   | Susceptible          | 0.062      | None*     | SDR      | PUD             |
| OC912   | Intermediate         | 0.5        | None*     | MDR      | PUD             |
| OC913   | Susceptible          | 0.125      | None*     | MDR      | PUD             |
| OC937   | Susceptible          | 0.125      | None*     | SDR      | CG              |
| OC939   | Resistant            | 4          | None*     | MDR      | PUD             |
| OC975   | Susceptible          | 0.125      | None*     | MDR      | IM              |
| OC985   | Susceptible          | 0.125      | None*     | MDR      | CG              |
| OC1031  | Susceptible          | 0.062      | None*     | MDR      | CG              |

SDR, single-drug resistance; MDR, multidrug resistance; CG, chronic gastritis; PUD, peptic ulcer disease; IM, intestinal metaplasia

*None, no specific variation detected as compared with genes from clarithromycin-sensitive *H. pylori* isolates

°ND, not determined (the obtained sequence was not appropriate for mutational analysis)

°N, not resistant to all antibiotics tested
Table 8. Frequency and distribution of virulence genotypes in relation to antibiotic resistance patterns among H. pylori isolates used in this study.

| Virulence Genotypes | MNZ S | CLR R | AMX S | CIP S | LEV R | RIF R | TCN R | Resistance No. (%) | Total No. (%) |
|---------------------|------|------|------|------|------|------|------|-------------------|--------------|
| cagL+               | 6   | 26  | 15   | 3    | 9.1  | 12.36| 22.66| 30.33 | 30.33 | 32.33 (97) |
| cagL-               | 0   | 1   | 3    | 9    | 1.3  | 0    | 0    | 0     | 0     | 1/3 (3)   |
| cagA+               | 4   | 12.1| 15   | 4.5  | 3.9  | 11.33| 20.6 | 9.27  | 27.3  | 7.27 (28.7)|
| cagA-               | 2   | 6.1 | 3    | 9    | 1.3  | 1   | 3   | 4.12  | 0     | 0.12 (1.2) |
| EPIYA motifs        |     |     |      |      |      |      |      |       |       | 4/33 (1.2) |
| ABC                 | 2   | 6.9 | 18   | 62.1 | 9    | 31   | 2   | 6.8   | 9    | 31 (16.52) |
| ABCC                | 1   | 3   | 1    | 3.4  | 2   | 6.9  | 0   | 0    | 2   | 6.9 (4.12) |
| ABCCC               | 0   | 1   | 3.4  | 0    | 0   | 1    | 3.4 | 0     | 1    | 3.4 (1.34) |
| Mixed type         | 1   | 3.4 | 5    | 17.2 | 4   | 13.8 | 1   | 3.4  | 4   | 13.8 (2.69) |
| vacA alleles        |     |     |      |      |      |      |      |       |       |            |
| vacA s1m1           | 3   | 9.1 | 3    | 9.1  | 2   | 12.1| 2   | 12  | 12  | 2 (21.2) |
| vacA s1m2           | 2   | 6.1 | 14   | 42.4 | 9   | 27.3 | 0   | 0    | 7   | 21.2 (12.36) |
| vacA s2m2           | 1   | 3   | 4    | 12.1 | 3   | 9.1  | 1   | 3   | 2   | 6.1 (2.61) |
| Oip “on”            | 4   | 15.4| 19   | 73.1 | 11  | 42.3 | 2   | 7.7  | 10  | 38.5 (18.69) |
| Oip “off”           | 0   | 3   | 11.5 | 3.8  | 1   | 3.8  | 1   | 3.8  | 2   | 7.7 (3.15) |
| habc2               | 6   | 18.2| 27   | 81.8 | 18  | 54.5 | 3   | 9.1  | 12  | 36.4 (23.69) |
| sabf                | 4   | 12.1| 23   | 69.7 | 15  | 45.4 | 2   | 6.1  | 10  | 30.3 (19.57) |
| sabf+               | 2   | 6.1 | 4    | 12.1 | 3   | 9.1  | 1   | 3   | 2   | 6.1 (4.12) |
| duft                | 4   | 12.1| 25   | 75.7 | 16  | 48.5 | 3   | 9.1  | 10  | 30.3 (20.66) |
| duft+               | 2   | 6.1 | 2    | 6.1  | 2   | 6.1  | 3   | 9.1  | 1   | 3 (4.12) |
| cagPAI integrity    |     |     |      |      |      |      |      |       |       |            |
| Intact cagPAI       | 3   | 9.4 | 18   | 56.2 | 11  | 34.4 | 2   | 6.2  | 8   | 25 (18.56.2) |
| Partial cagPAI      | 3   | 9.4 | 8    | 25   | 6   | 18.7 | 1   | 3.1  | 4   | 12.5 (4.12) |
| Totally deleted cagPAI | 0  | 1   | 3    | 3    | 0   | 1    | 3   | 0    | 1   | 3 (1.30) |

MNZ, metronidazole; CLR, clarithromycin; AMX, amoxicillin; CIP, ciprofloxacin; LEV, levofloxacin; RIF, rifampicin; TCN, tetracycline; S, susceptible; I, intermediate; R, resistant

*Denotes the presence of multiple cagA EPIYA motifs indicating mixed infections