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Original article

Genetic Diversity and Drug Resistance of *Helicobacter pylori* Strains in Isfahan, Iran

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Abstract

Objective(s)
Resistance to antimicrobial agents, particularly metronidazole and clarithromycin, is frequently observed in *Helicobacter pylori* and may be associated with treatment failure. This resistance rate varies according to the population studied. The aim of this study was to assess the pattern of antimicrobial resistance of *H. pylori* isolates from dyspeptic patients in Isfahan.

Materials and Methods
Antral gastric biopsies from 230 dyspeptic patients were cultured. Susceptibility testing to commonly used antibiotics performed on pure cultures of 80 *H. pylori*-positive isolates by Modified Disk Diffusion Method (MDDM). Genomic DNA extracted and subjected for study of entire genomic pattern using Random Amplified Polymorphic DNA- Polymerase Chain Reaction (RAPD-PCR).

Results
The overall rates of primary resistance were 30.0%, 8.75%, 6.25%, 3.75%, 3.75%, and 2.50% for metronidazole, ciprofloxacin, clarithromycin, azithromycin, tetracycline, and amoxicillin, respectively. Multiple antibiotic resistances were observed in 8 of 27 resistant isolates (29.6%) that mainly were double resistance with the prevalence of 6.25%. No association between antimicrobial resistance and either the gender, age or clinical presentation of the patients were detected. In RAPD-PCR, great diversity observed in 27 resistant strains isolated from different patients and this heterogeneity was not significantly different from susceptible strains.

Conclusion
Primary *H. pylori* resistance to metronidazole in our population was lower than the developing world and even other parts of Iran, to ciprofloxacin was considerable in comparison with results in most other countries. Moreover, antibiotic resistance had no effect on genomic pattern of *H. pylori* isolates. Finally, pretreatment *H. pylori* isolates susceptibility testing is highly recommended.

Keywords: Drug resistance, *Helicobacter pylori*, Iran, Random Amplified Polymorphic DNA- Polymerase Chain Reaction

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Introduction

Helicobacter pylorus is one of the most common chronic bacterial infections worldwide. Association of *H. pylori* colonization of stomach with chronic gastritis, peptic ulcer and gastric malignancies has been well documented (1). Although *H. pylori* is susceptible to many antimicrobials *in vitro*, clinical experience has demonstrated that *H. pylori* infection is not easy to cure *in vivo* (2, 3). Triple therapy combining a proton pump inhibitor with two antibiotics, e.g. clarithromycin, metronidazole or amoxicillin, represents the standard in *H. pylori* eradication regimens (4, 5). Although this treatment is successful in 70-90% of cases (6, 7), treatment failure can be resulted from the presence of antimicrobial–resistant strains of *H. pylori* (6, 8, 9). The prevalence of clarithromycin, metronidazole, and amoxicillin resistances varies between countries and is highest for metronidazole (10, 11). Resistance to tetracycline and ciprofloxacin has been reported but appears uncommon (9, 11). Various *in vitro* susceptibility methods have been used: Agar dilution, E-test and disk diffusion. No reference method has been formally proposed for testing *H. pylori* strains against antibiotics (2), nevertheless, agar dilution is considered as the gold standard by NCCLS. However, it is time consuming and labor intensive (2, 5, 12, 13). The E-test has been recommended for *H. pylori* and gives MIC results comparable to those of the agar dilution method, but it is expensive, which limits its use (11, 14). Disk diffusion method is commonly used to test the susceptibility in any routine laboratory setting because it is simple and cheap (15, 16). Moreover, this procedure is practical, accurate, and clinically applicable (14). Thus, reliable, easy–to-perform, fast and inexpensive susceptibility testing methods are required for routine purposes (19, 20). *H. pylori* isolates obtained from different individuals show substantial genomic diversity (21, 22). The random amplified polymorphic DNA (RAPD) PCR, method has been valuable for studies of *H. pylori* genetic diversity and transmission (23). It is proved that RAPD–fingerprinting is an efficient, reliable and sensitive method of distinguishing between clinical isolates of *H. pylori* (24-26).

Genetic diversity in *H. pylori* may result in phenotypic variation between different strains, including susceptibility to antimicrobials (20). As antimicrobial resistance has become an increasing problem that may soon compromise the effectiveness of current therapies, thus each country must provide local data to guide treatment policies (3, 8).

The purposes of the present study were: (ì) to assess the prevalence of primary resistance to commonly used antibiotics among *H. pylori* isolates in the central part of Iran, Isfahan, (ìì) to evaluate the genetic diversity pattern of resistant strains by RAPD-fingerprinting and comparison of their pattern with the sensitive strains.

Materials and Methods

Patients and specimens

Antral gastric biopsy samples were obtained from 230 patients who had gastrointestinal diseases, between January and June 2006 at the Department of Gastroenterology of Al-Zahra Hospital in Isfahan, Iran. This study was approved by the local Ethics Committee. The clinical diagnosis of the patients was established by endoscopy. None of the patients had received previous treatment for the eradication of *H. pylori*.

Isolation and identification of H. pylori

Each biopsy specimen was processed and cultured as described by Lang and Garcia (20). After 3-5 days of incubation at 37 ºC in microaerobic conditions (MART, ANOXOMAT, Lichtenvoorde, Netherlands), bacterial growth was identified as *H. pylori* on the basis of its colony morphology on plated media, gram stain reaction, and positive biochemical reactions to urease, catalase and oxidase tests. Individual cultures representing colonies from each patient was frozen at -70 ºC in *Brucella* broth (Quelab, UK) containing 20% glycerol and 10% Fetal-calf

serum (FCS) (Bahar Afshan -Iran) until susceptibility testing and RAPD-PCR reaction were performed.

Antibiotic susceptibility testing

For this purpose, Modified Disk Diffusion Method (MDDM) was used. Stored isolates of *H. pylori* were regrown on Columbia agar (Merck, Germany) supplemented with 5% sheep blood, 10% FCS, and 1 camp selectatab tablet (MAST Diagnostics, UK) containing vancomycin 10 mg, polymyxin B 2500 IU, and trimethoprim 5 mg. A suspension from 3-day-old bacterial cells of each isolate was prepared in *Brucella* broth (2 ml) equivalent to the McFarland turbidity standard 3-4. The suspensions were spread on to the surface of the Columbia agar base with 5% sheep blood and 10% FCS with sterile cotton swabs. The plates briefly dried and then antibiotic disks (Mast Diagnostics, UK) including metronidazole (5 µg), azithromycin (15 µg), clarithromycin (15 µg), ciprofloxacin (5 µg), amoxicillin (10 µg) and tetracycline (30 µg) were added two per plate and incubated microaerobically at 37 ºC for 3 days. One plate without antibiotic disk was used as a control. The inhibition zone diameters measured in millimeters, with a ruler. Resistance determined by a zone of growth inhibition <16 mm corresponding to an MIC>8 mg/L for metronidazole, zone diameters ≤30 mm for clarithromycin or azithromycin, ciprofloxacin and tetracycline (9, 11, 27) and zone diameter ≤25 mm for amoxicillin (20). For metronidazole the nonsusceptible categories of intermediate (16-20 mm) and resistant combined into a single "resistant" category. Greater zones of complete growth inhibition indicated the presence of susceptible strains. The procedure repeated for cultures that were defined as resistant. *H. pylori* strain ATCC 26695 used as a reference strain for the quality control of antibiotic susceptibility testing.

Preparation of genomic DNA

After recovery of frozen *H. pylori* isolates as described above, the bacteria were grown with gentle shaking at 70 rpm (Kuhner shaker, Switzerland) under microaerobic conditions at 37 ºC for 24 hr in 10 ml *Brucella* broth containing 1 ml FCS. Also, to rule out the possibility of contaminating bacteria, vancomycin (10 µg/ml) and trimethoprim (5 µg/ml) were added. Liquid cultures centrifuged at 8000 g for 5 min, and then pellets washed twice with phosphate-buffer saline (PBS, pH=7.2) and once with Tris-HCl buffer containing ethylene diamine tetraacetate (TE, pH=8). The pellets were suspended in 200 µl TE buffer.

Chromosomal DNA from each *H. pylori* strain prepared using the guanidium thiocyanate method (28). The concentration and quality of DNA preparations determined spectrophotometrically by measuring absorbance of A_{260}, A_{280}, A_{230} and by agarose gel electrophoresis with DNA standard (λ DNA marker, Genecraft, Germany).The DNA samples used had an A_{260}/A_{280}, and A_{230}/A_{280} of ≥1:8. The DNA preparations stored at -20 ºC.

RAPD-PCR fingerprinting

RAPD fingerprinting was undertaken using 2 primers: 1254 (5′-CCGCAGCCAA-3′) and 1281 (5′-AACGCGCAAC-3′) according to the method by Akopyanz *et al* (29). The PCR reactions carried out in 25 µl volumes containing 20 ng of *H. pylori* genomic DNA, 3 mM $MgCl_2$, 20 pmoles of each primer, 2.0 U of Ampli Taq DNA polymerase, and 0.25 mM (each) deoxy nucleotiede triphosphate (dNTP) in standard PCR buffer. All the reagents obtained from the Amplisense, biotechnologies. The reaction conditions performed as described by Akopyanz *et al* (29). The PCR amplicons analyzed by 2% agarose gel electrophoresis and photographed under ultraviolet light. A negative control (PCR reagents without DNA template) and positive control (strain ATCC 26695) were also used. The 1 Kb DNA ladder (Genecraft, Germany) used as a size marker (M) in all gels. Only the major RAPD bands considered.

Statistical analyses

Data analyzed in SPSS version 15. Categorical variables compared by Pearson chi-square test and Fisher's exact test. The 95.0% confidence interval calculated and $P<0.05$ considered as statistically significant.

Results

H. pylori isolates in various clinical groups

Of the 230 patients studied, 80 (34.8%) found to harbour *H. pylori* in antrum of the stomach. *H. pylori* was present in 30/93 (32.2%) gastritis patients as compared with 9/24 (37.5%) gastric ulcer, 21/45 (46.6%) duodenal ulcer, 2/4 (50.0%) gastric cancer and 18/64 (28.0%) non-ulcer dyspepsia patients. Of these *H. pylori* positive cases, were 43 (54.0%) male and 37 (46.0%) female.

Prevalence of primary H. pylori resistance

Antimicrobial resistance of 80 *H. pylori* isolates to six drugs, as determined by MDDM is listed in Table 1.

Table 1. Primary and combined resistance of *H. pylori* isolates to antimicrobial agents.

Antibiotics	n (%) of resistant isolates		
	S	I	R
MTZ	56 (70.0)	3 (3.75)	15 (18.7)
CIP	73 (91.2)		1(1.25)
MTZ+CLA+ATH+AMX+TET+CIP			1(1.25)
MTZ+CLA+ATH+TET+CIP			1(1.25)
CLA+AMX+TET+CIP			1(1.25)
CLA+ATH			1(1.25)
MTZ+CLA			1(1.25)
MTZ+CIP			3 (3.75)

MTZ: Metronidazole, CIP: Ciprofloxacin, CLA: Clarithromycin, ATH: Azithromycin, AMX: Amoxicillin, TET: Tetracycline, S: Susceptible, I: Intermediate, R: Resistant

Overall, of the 80 pretreatment *H. pylori* isolates, 27 (33.7%) strains resisted to at least one of the antimicrobial agents. The highest resistance rate was for metronidazole (n=21, 26.2%), whereas 3 isolates (3.75%) were intermediate. Therefore, the overall rate of primary resistance to metronidazole was 30.0%. The resistance rates to other antibiotics in *H. pylori* isolates recorded as follows: ciprofloxacin 7 (8.75%), clarithromycin 5 (6.25%), azithromycin 3 (3.75%), tetracycline 3 (3.75%) and amoxicillin 2 (2.50%). In 8 of 27 (29.6%) resistant cases, combined resistance was found.

Among these multiple resistances, the maximum resistance rate observed in the case of metronidazole plus ciprofloxacin (n=3, 3.75%). One case resisted to all the six drugs tested (Table 1).

In this study, no resistance observed as colonies grew within the inhibitory zones in all antibiotic disks. Statistical analysis revealed that resistance rate to either antibiotic in *H. pylori* isolates was not significantly associated with the gender of patients ($P= 0.647$). Also, there were no differences ($P= 0.848$) in the antimicrobial resistance rates and diseases (peptic ulcer including gastric ulcer, duodenal ulcer, gastritis and non-ulcer dyspepsia) or different age groups (patients 18-40, 41-60 and >60 years old) ($P=0.645$).

RAPD-PCR patterns of H. pylori isolates

RAPD-PCR resulted in distinct bands (2-11 and 1-13 bands for primers 1254 and 1281, respectively) of strong intensities (Figure 1). The bands ranged in size from 500 bp to 4000 bp for both primers. Each primer resulted in a unique pattern for each strain, different from all other. Common bands appeared to be present among DNA fingerprints produced by some isolates. The reproducibility of RAPD patterns was tested using 6 different isolates of both primers. All the patterns obtained were

identical, thus confirming the stability of the RAPD-PCR method with the same isolates.

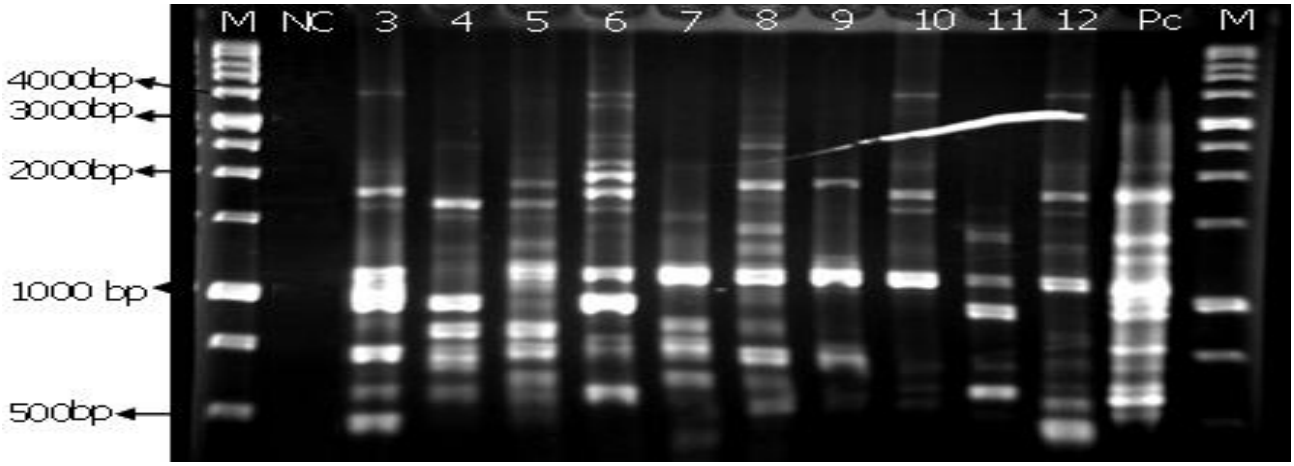

Figure 1a. RAPD-PCR fingerprinting of clinical isolates of *H. pylori* using primer 1254 from 10 patients with gastrointestinal diseases.
Lane 1 and 14: DNA Ladder 1kb (M), Lane 2: Negative control (Nc), Lane 3-5, 7, 8, 11 and 12: Sensitive strains
Lane 6, 9 and 10: Resistant strains to metronidazole, Lane 13: Positive control- *H. pylori* ATCC 26695 (Pc).

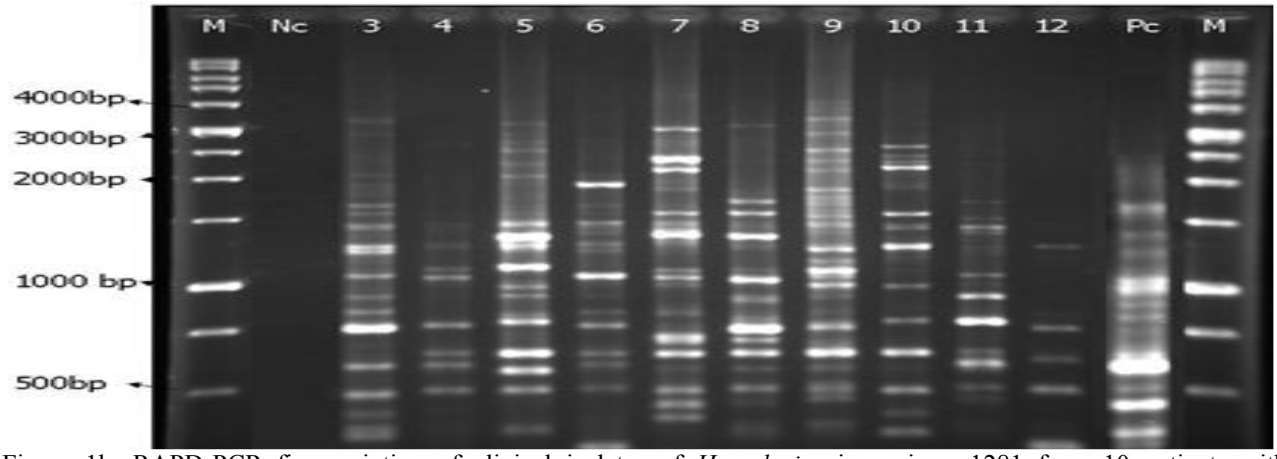

Figure 1b. RAPD-PCR fingerprinting of clinical isolates of *H. pylori* using primer 1281 from 10 patients with gastrointestinal diseases.
Lane 1 & 14: DNA Ladder 1 kb (M), Lane 2: Negative control (Nc), Lane 3, 4, 6, 7, 9, 11 and 12: Sensitive strains, Lane 5, 8 and 10: Resistant strains to metronidazole, Lane 13: Positive control- *H. pylori* ATCC 26695 (Pc).

Discussion

H. pylori infects the majority of the adult population in developing countries including Iran. The rate of infection in Iranian adults according to serology data is up to 80% (30). *H. pylori* infection is difficult to cure and requires combination therapies for eradication. Many *H. pylori* strains show resistance to one or more antimicrobial agents *in vitro* and this may be the cause of eradication failure (8). Moreover, patient noncompliance and the location of the bacterium which is beneath the gastric epithelium, are involved in the treatment failure (2, 31, 32). Resistance to antibiotics among *H. pylori* isolates is prevalent worldwide and varies widely by geographic location and socioeconomic status (2, 33, 34). However, it is mentioned elsewhere that reduced socio-economic status appears not to be associated with *H. pylori* antimicrobial resistance (12). Metronidazole is an important antimicrobial used in the treatment of *H. pylori* infection (13, 31). Determination of strain susceptibility to antibiotics, particularly to metronidazole, is very essential (16). In the current study, antibiotic susceptibility testing was applied on *H. pylori* isolates by Modified Disk Diffusion Method (MDDM). This method is reliable when the procedures are standardized (16).

Although The NCCLS has proved the agar dilution method as the test of choice and reference for antimicrobial susceptibility testing of *H. pylori*, however it is a laborious and time-consuming method and difficult to evaluate (2, 13, 16, 35). Moreover, it is documented in several studies that there is a good correlation between MDDM and E-test methods and both are very good alternatives to agar dilution (11, 13, 14, 17, 18, 20). Also, Chaves *et al*, Xia *et al* and Decross *et al* have shown that disk diffusion method is a good alternative for determining antibiotic susceptibility of *H. pylori*, particularly to metronidazole (14, 16, 36).

The prevalence rate of metronidazole resistance among *H. pylori* strains is highly variable and is higher in developing countries than in developed countries (2). This difference may be due to the general use of metronidazole in developing countries for other infectious problems, such as protozoal diseases (2, 20, 31, 37). In our analysis, the overall resistance to metronidazole had the highest rate (24/80, 30%).

In agreement with previous studies, we also calculated the inhibition zones resistant (<16 mm) and intermediate (16-20 mm) as resistant strains (3, 11, 14, 16, 30). The narrow range of MICs and homogenous susceptibility of *H. pylori* to clarithromycin, ciprofloxacin and tetracycline have been reported; therefore, large inhibitory areas (>30 mm) seem to be most suitable for detecting isolates which are susceptible to all drugs except metronidazole (9). The resistance rate to metronidazole in our study is comparable with the previous studies from Italy (38), Sweden (39), Netherlands (40), Bulgaria (9), Lebanon (41) and United States (42). However, the resistance rate to metronidazole in our investigation (30%) was lower than those reported by Falsafi *et al* (72-79%), Fallahi *et al* (54.16%) and Roghani *et al* (42%) (43- 45).

Such discrepancy in the rate of prevalence within Iran is seen also in Taiwan and Alaska (8, 34). Reasons for this regional difference are unclear but may include local antibiotic prescription patterns and methodological variations in the susceptibility testing of *H. pylori* (5, 41).

The resistance rate for ciprofloxacin, clarithromycin, azithromycin, tetracycline and amoxicillin were 8.75%, 6.25%, 3.75%, 3.75% and 2.50%, respectively. Interestingly, in comparison with the low prevalence (≤1%) of ciprofloxacin resistance in most studies (46), our results showed a remarkably (8.75%) resistance rate. The data is similar to the previous reports from Bulgaria and India with prevalence rates of 8.6% and 12%, respectively (9, 11).

In industrialized countries approximately 10% of the *H. pylori* strains are clarithromycin-resistant (2, 47), but in developing countries this resistance rate is higher, varying between 25% to 50% (47). With regard to resistance rate to clarithromycin (6.25%), similar results in agreement with our study have shown by Kim *et al* and Van der Ende A (48, 49). However, in several studies conducted in Iran, the resistance rates to clarithromycin were 8.32%, 16.7%, 16%, 5.9% in children, and 2.4% in adults (30, 44, 50, 51). Tetracycline resistance has been reported but it is found in a very low percentage of strains and reports corresponding to amoxicillin resistance are infrequent (2, 11, 20), that is consistent with our results. In this investigation, 29.6% of the resistant isolates exhibited multiple resistances (mainly double resistance with prevalence of 6.25%) to different drugs. Other authors have shown 12%, 9.3% and 33.8% of these multiple resistances (8, 9, 11). It is mentioned that such strains have been associated with therapy failure (33). Primary antibiotic resistance rates demonstrated no association with age, gender and clinical diseases (peptic ulcer, gastritis and non-ulcer dyspepsia). In several studies from Bulgaria (9), Germany (10), Costa Rica (20), UK (27) and Iran (44, 50) similar results were reported.

The reason for great diversity within *H. pylori* clinical isolates may be due to its different genotypic patterns and phenotypic diversity such as differences in drug resistance, adherence specificity, cytotoxin production as well as CagA expression (52). In

the work of Dore *et al* (3), great diversity was observed in resistant *H. pylori* isolated from different patients by REP-PCR and each isolate produced an amplified DNA pattern of 15-20 major bands. In another study in Germany, no association was found between *H. pylori* genotypes and antibiotic resistance phenotypes within related patients (4). In our RAPD-fingerprinting, the diversity of resistant *H. pylori* strains was exhibited by a unique band pattern in each of the clinical isolates. Such patterns were also found in sensitive strains.

Conclusion

With regards to marked variation in the level of antibiotic resistance amongst *H. pylori* strains between different countries, therefore, it is important to carry out susceptibility studies in each population. On the other hand, it seems antibiotic resistance has no effect on the genomic pattern of *H. pylori* strains and each isolate represents its exclusive pattern, irrespective of the existence of antibiotic resistance.

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