



Antibiotic resistance and *cagA* gene correlation: A looming crisis of *Helicobacter pylori*

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Abstract

AIM: To determine antibiotic resistance of *Helicobacter pylori* (*H. pylori*) in Pakistan and its correlation with host and pathogen associated factors.

METHODS: A total of 178 strains of *H. pylori* were isolated from gastric biopsies of dyspeptic patients. Susceptibility patterns against first and second-line antibiotics were determined and trends of resistance were analyzed in relation to the sampling period, gastric conditions and *cagA* gene carriage. The effect of *cagA*

gene on the acquisition of resistance was investigated by mutant selection assay.

RESULTS: The observations showed that monoresistant strains were prevalent with rates of 89% for metronidazole, 36% for clarithromycin, 37% for amoxicillin, 18.5% for ofloxacin and 12% for tetracycline. Furthermore, clarithromycin resistance was on the rise from 2005 to 2008 (32% vs 38%, $P = 0.004$) and it is significantly observed in non ulcerative dyspeptic patients compared to gastritis, gastric ulcer and duodenal ulcer cases (53% vs 20%, 18% and 19%, $P = 0.000$). On the contrary, metronidazole and ofloxacin resistance were more common in gastritis and gastric ulcer cases. Distribution analysis and frequencies of resistant mutants *in vitro* correlated with the absence of *cagA* gene with metronidazole and ofloxacin resistance.

CONCLUSION: The study confirms the alarming levels of antibiotic resistance associated with the degree of gastric inflammation and *cagA* gene carriage in *H. pylori* strains.

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Key words: *Helicobacter pylori*; Antibiotic resistance; *cagA*; Pakistan; Clarithromycin; Metronidazole; Fluoroquinolones

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is among the most widespread infectious agents because of its high colonization rate and persistent nature in its host's stomach. Mostly the colonization is silent but overt damage of gastric mucosa occurs in certain cases leading to the development of gastritis, duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC) and mucosa associated lymphoid tissue lymphoma. It is believed that CagA toxin is responsible for the underlying virulence mechanism because of its ability to translocate into gastric epithelial cells. In host cells, it binds with cellular SRC homology 2 domain-containing tyrosine phosphatase (SHP-2) protein and subsequently damage of gastric mucosa.

Triple therapy constituting the combination of a proton pump inhibitor or bismuth citrate and two antibiotics such as amoxicillin (AML), clarithromycin (CLR) or metronidazole (MTZ) is the internationally recommended first-line regime to eradicate *H. pylori* in symptomatic patients^[1,2]. However, the combination might be delivered concomitantly, sequentially or in the form of a traditional straight course for 10-15 d to attain the highest cure rate^[3]. In case of treatment failure, several other antibiotics such as fluoroquinolones and tetracycline (TE) are used as secondary options^[3,4]. The increased rates of CLR and MTZ resistance have further compounded the already challenging treatment strategy within the harsh acidic environment of the stomach. Patient compliance has declined to less than 80% due to resistance against one of the antibiotics used in the first-line regime^[5,6].

Pakistan is among the countries with high prevalence of *H. pylori* infection. The organism is not only associated with severe clinical outcomes^[7] but is also carried by the healthy population. Empirical treatment has always been in practice without examining whether it matches *in vitro* antibiotics susceptibility testing (AST) or not. As a result, patient compliance has diminished up to 70%-75% in the last decade^[8,9]. As most of studies from Pakistan are based on the outcome of therapy, information is scanty on the status of AST profiles of local isolates. Previous attempts, some of which are based on alternate AST methods, provide limited information^[10,11], while no data is available on AST profiles for second-line options.

The paucity of information on the issue and the key role it plays in controlling *H. pylori* infection led us to conduct the present investigation that not only provides a detailed AST profile of local *H. pylori* isolates against first- and second-line regimes but also analyzes their distribution in various groups of patients. The study also contributes toward the better understanding of the role of *cagA* gene in the evolution of resistance.

MATERIALS AND METHODS

Patients and sampling

A total of 178 *H. pylori* strains isolated from gastroduodenal biopsies were included in this study. Biopsy samples were taken from symptomatic patients ($n = 450$) who

underwent gastroduodenal endoscopy at Medical Unit II, Civil Hospital, Dow University of Health Sciences, Karachi, from March 2005 to November 2008. They were grouped as non ulcerative dyspepsia (NUD), gastritis, GU and DU on the basis of endoscopic findings. Patients with previous treatment history for *H. pylori* infection and/ or GC were excluded. Samples were collected in 20% sterile glucose solution, transported in ice, and processed within two hours of collection. The study was conducted upon approval from the ethical review board of the University of Karachi, Pakistan.

Molecular diagnosis

Genomic DNAs were extracted from crushed tissue samples by SDS-PK method^[12] (Khan, 2006 No. 354). Molecular diagnosis for the presence of *H. pylori* was conducted by polymerase chain reaction (PCR) targeting the *16SrRNA* gene as described previously^[13]. Samples that were found positive with the *16SrRNA* gene of *H. pylori* were further examined for the presence of the *cagA* gene by PCR using primers designed for the entire 3' repeat conserved region. Amplification was performed at 35 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min with a final extension of 7 min at 72 °C^[14]. A segment of human β -globulin gene was amplified as the internal control.

Isolation and identification of *Helicobacter pylori* strains

For the isolation of *H. pylori*, biopsy samples were crushed with the help of a sterile disposable tissue homogenizer and inoculated on Columbia Blood Agar (Oxoid, United Kingdom) containing 7% laked horse blood (Oxoid, United Kingdom) and *H. pylori* selective supplement Dent (Oxoid, United Kingdom). Plates were incubated at 37 °C for 5 d under microaerophilic conditions using a Campygen gas generating kit (Oxoid, United Kingdom). Suspicious small dew drop colonies were subjected for morphological and biochemical identification.

For further confirmation, genomic DNA was extracted and subjected to PCR analysis for the *16SrRNA* gene of *H. pylori* per the above-mentioned protocol.

Determination of minimum inhibitory concentrations of antibiotics

Susceptibility patterns of *H. pylori* isolates was determined against a battery of antibiotics including MTZ, AML, CLR, DA, TE, ofloxacin (OFX) and erythromycin (E). Various concentrations of antibiotics were added to Mueller-Hinton agar containing 5% old sheep blood (SB-MHA). Bacterial suspensions were prepared in sterile phosphate buffered saline (PBS) with density equivalent to 3 McFarland's turbidity standard. Ten microliters of each strain was spotted on the plates and incubated for 72 h under microaerophilic conditions. The lowest concentration of antibiotic able to inhibit visible bacterial growth was considered as minimum inhibitory concentrations (MIC). Results were interpreted according to standard criteria^[15].

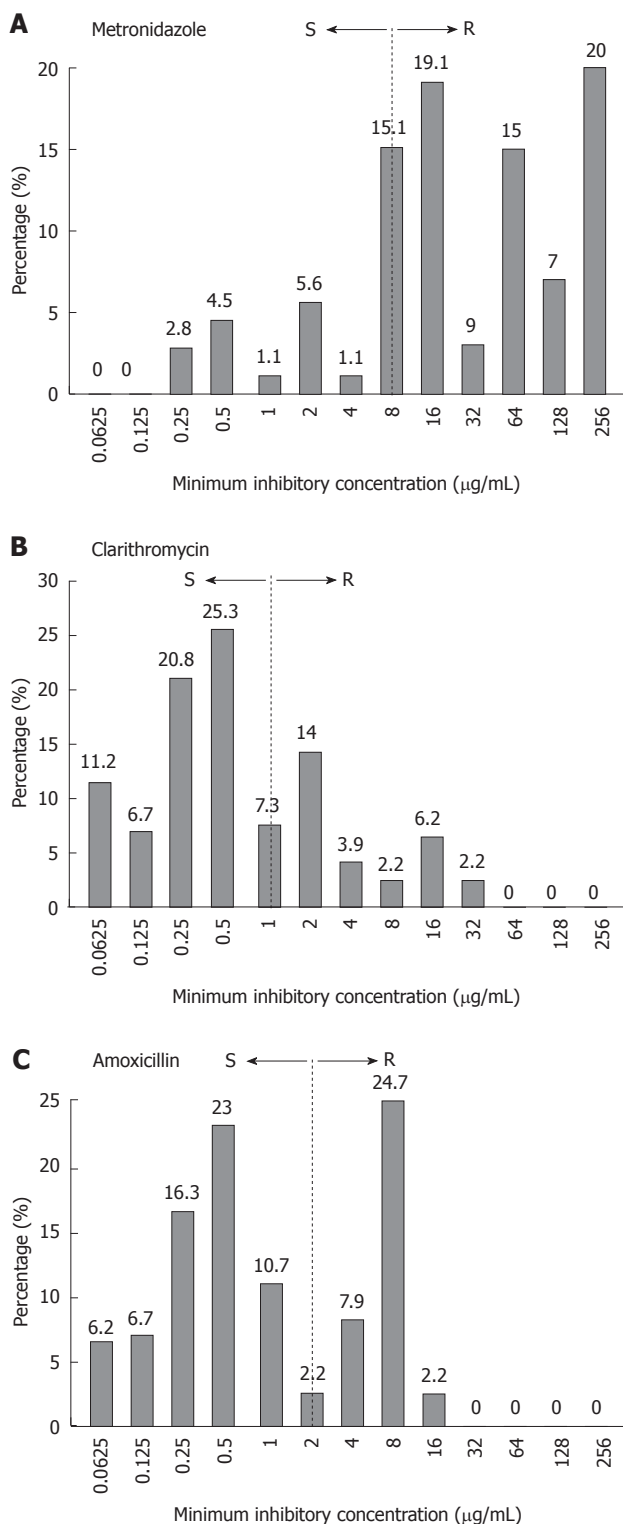


Figure 1 Prevalence of *Helicobacter pylori* resistance to first-line drugs. Dotted box represents the distribution of resistant strains at different minimum inhibitory concentrations (MICs). MIC breakpoints were ≥ 8 µg/mL (A), ≥ 1 µg/mL (B) and ≥ 2 µg/mL (C). S: Sensitive; R: Resistant.

Mutant selection assay

To determine the role of *cagA* gene in the emergence of resistance, two *cagA*⁺ and two *cagA*⁻ strains were subjected to mutant selection assay. A total of 1.2×10^6 – 1.5×10^6 colony forming units (CFU) of bacterial strains suspended in PBS was spread over SB-MHA plates contain-

ing varying concentrations of antibiotics and incubated for 72 h at 37 °C under microaerophilic conditions. Frequency of resistant mutants was determined as the CFU of each strain grown on antibiotic supplemented plates divided by the starting inocula^[16].

Statistical analysis

Statistical analyses were performed by PASW statistics 18 (SPSS Inc., Chicago, IL, United States). Pearson's χ^2 test was applied to compare categorical data. Linear regression was applied to correlate the frequency of resistance with *cagA* gene. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Antibiotic susceptibility profile

Out of 450 dyspeptic patients, 201 (45%) were found positive for *H. pylori* by PCR and 178 (40%) by culture. AST profile of 178 *H. pylori* strains revealed high levels of resistance against the first-line regime. A total of 149 (84%) were found to be resistant (MIC ≥ 8 µg/mL) to MTZ. As shown in Figure 1A, 34 (19%) strains had MIC 16 µg/mL, 16 (9%) had 32 µg/mL, 27 (15%) had 64 µg/mL, 12 (7%) had 128 µg/mL and 35 (20%) had MIC 256 µg/mL whereas 27 (15%) isolates were inhibited at boarder-line concentration 8 µg/mL. In case of CLR, 64 (36%) strains showed resistance with MICs ≥ 1 µg/mL. The highest MIC 32 µg/mL was observed only in 4 (2.2%) isolates while 25 (14%) had MIC 2 µg/mL (Figure 1B). Sixty six (37%) strains showed resistance to AML with MICs ≥ 2 µg/mL. Surprisingly, 44 (24.7%) strains exhibited MIC 8 µg/mL (Figure 1C).

Emergence of resistance against first-line therapy led us to investigate the susceptibility patterns of antibiotics usually given as second-line treatment options, such as TE, fluoroquinolones and clindamycin (DA). Data analysis shows an evenly distributed pattern of DA activity with MICs ranging from 0.25 to 32 µg/mL (Figure 2A). Resistance to TE was found in 21 (12%) strains. MIC 4 µg/mL was observed in 15 (8.4%) strains and 8 µg/mL in 7 (3.9%) strains (Figure 2B). As shown in Figure 2C, a total of 33 (18.5%) strains were resistant to OFX. MIC of OFX was 2 µg/mL and 4 µg/mL for 21 (11.8%) and 12 (6.7%) strains respectively. We further analyzed the rate of multidrug resistance (MDR) in our studied population. A total of 46 (26%) of the isolates were resistant to two antibiotics i.e. MTZ and AML whereas 62 (35%) were resistant to MTZ and CLR. MDR isolates who were resistant to all first-line antibiotics (R-phenotype; MTZ^rCLR^rAML^r) were 20 (11%). Of these 7 (4%) were also resistant to tetracycline (R-phenotype; MTZ^rCLR^rAML^rTE^r) as shown in Figure 2D.

Correlation of resistance with demographic and disease factors

We next examined whether the proportion of strains showing antibiotic resistance were increased over time or not. For the purpose, the strains were divided into four groups; 2005 (*n* = 24), 2006 (*n* = 55), 2007 (*n* = 73) and

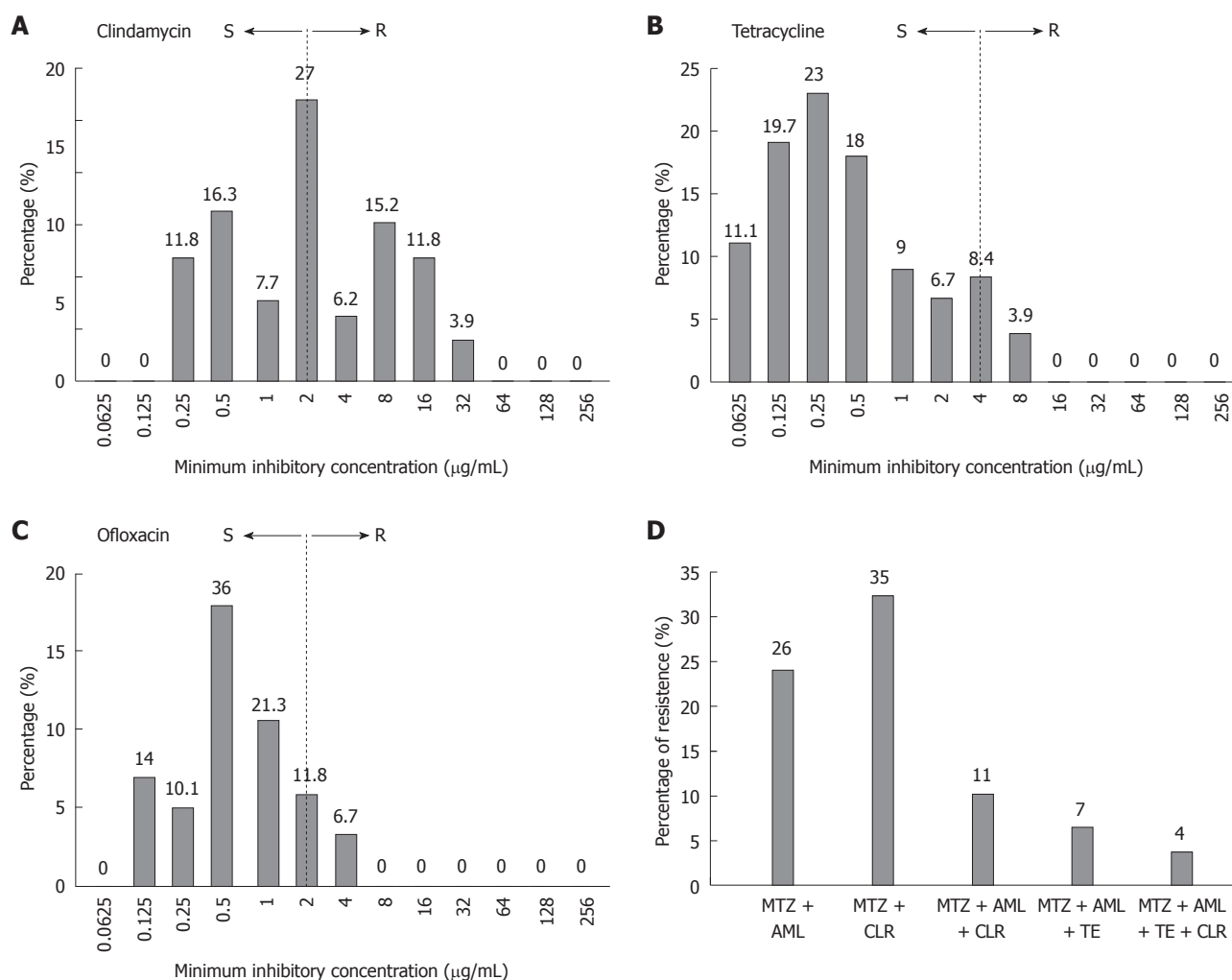


Figure 2 The prevalence of *Helicobacter pylori* resistance to second-line drugs. Dotted box represents the distribution of resistant strains at different minimum inhibitory concentrations (MICs). MIC breakpoints were ≥ 1 μg/mL (A), ≥ 4 μg/mL (B) and ≥ 2 μg/mL (C); D: Distribution of multidrug resistant strains. AML: Amoxicillin; CLR: Clarithromycin; MTZ: Metronidazole; TE: Tetracycline. S: Sensitive; R: Resistant.

2008 ($n = 26$) according to the year of sample collection. In general, a significantly progressive trend was only observed in CLR resistance from 32% ($n = 8$) in 2005 to 35% ($n = 19$) in 2006, 37.5% ($n = 27$) and 38% ($n = 19$) in 2008 ($P = 0.004$). On the contrary, MTZ resistant strains went down by year from 72% ($n = 17$) in 2005 to 69% ($n = 38$) in 2006, 68% ($n = 50$) in 2007 and 65% ($n = 17$) in 2008. However AML and OFX resistance remained steady with the rates of approximately 37% and 19% throughout the study period whereas overall resistance rates of TE were 10% ($n = 2$) in 2005, 13% ($n = 7$) in 2006, 12% ($n = 9$) in 2007 and 11% ($n = 3$) in 2008 (Figure 3A).

Comparative analysis was also performed according to the endoscopic findings of each patient from which *H. pylori* strains were isolated. Out of 178, a total of 25 strains were isolated from NUD cases, 89 gastritis cases, 26 GU cases and 38 from DU cases. Our findings indicate that AML and OFX resistance rates were more common in gastritis patients at 48% ($n = 43$) ($P = 0.005$) and 22% ($n = 20$) of cases ($P = 0.08$) respectively. In contrast, respective resistance rates of AML and OFX were 31%

($n = 8$) and 19% ($n = 5$) in GU and 16% ($n = 6$) and 21% ($n = 8$) in DU patients while no OFX resistant strain was found in NUD group. In case of MTZ, resistance rate was significantly higher among the patients with damaged mucosa such as gastritis (90%), GU (100%) and DU (92%) compared with NUD cases (70%) ($P = 0.001$). CLR resistance was observed in 53% ($n = 13$) of NUD, 20% ($n = 18$) gastritis, 18% ($n = 4$) GU and 19% ($n = 5$) of DU cases ($P = 0.000$) as shown in Figure 3B.

Correlation of drug resistance with *cagA* gene

The *cagA* genotypes of *H. pylori* usually correlate with the severity of disease; therefore, we determined the relationship of *cagA* gene with the susceptibility profile in local isolates. In this study 83 out of 178 (47%) *H. pylori* strains carried the *cagA* gene. The percentage of *cagA*⁺ strains was 49% ($n = 44$) among patients with gastritis, 69% ($n = 18$) GU and 60% ($n = 23$) DU whereas only 8% ($n = 2$) of the strains isolated from NUD cases carried this gene. Due to the low frequency of *CagA*⁺ strains in NUD cases, further analysis was only based only on a total of 153 strains which were isolated from gastritis, GU and DU

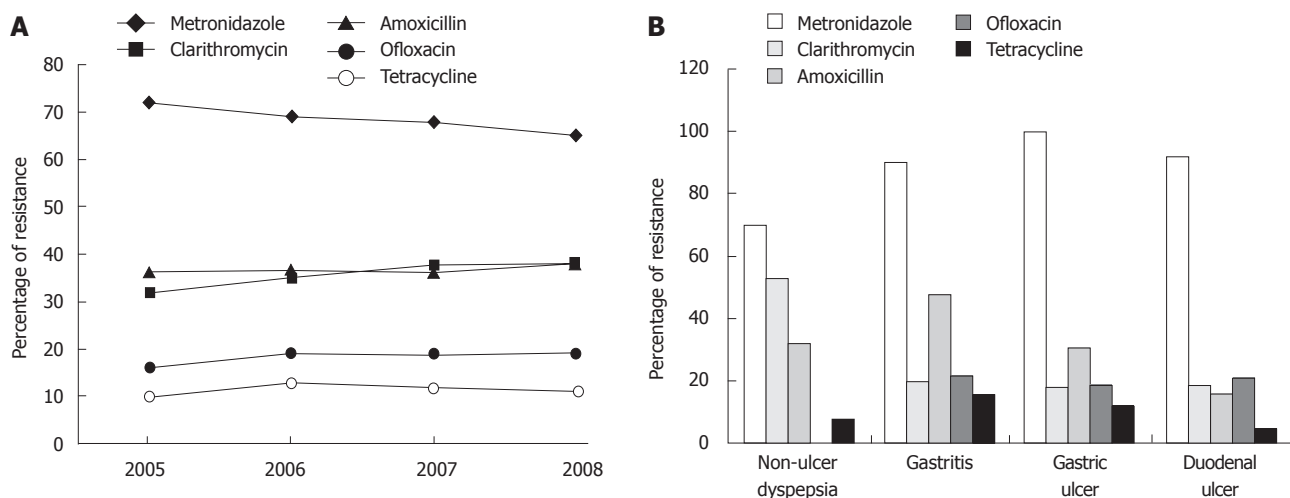


Figure 3 The trend analysis antimicrobial drug resistance in *Helicobacter pylori* strains ($n = 178$). A: From 2005 to 2008, significant increase in clarithromycin resistance was observed by linear regression ($P = 0.004$); B: Correlation of drug resistant strains in various groups of patients. Significant distribution of metronidazole ($P = 0.001$), clarithromycin ($P < 0.000$) and amoxicillin ($P = 0.005$) resistance was observed by Pearson's χ^2 test. NUD: Non ulcerative dyspepsia; GU: Gastric ulcer; DU: Duodenal ulcer.

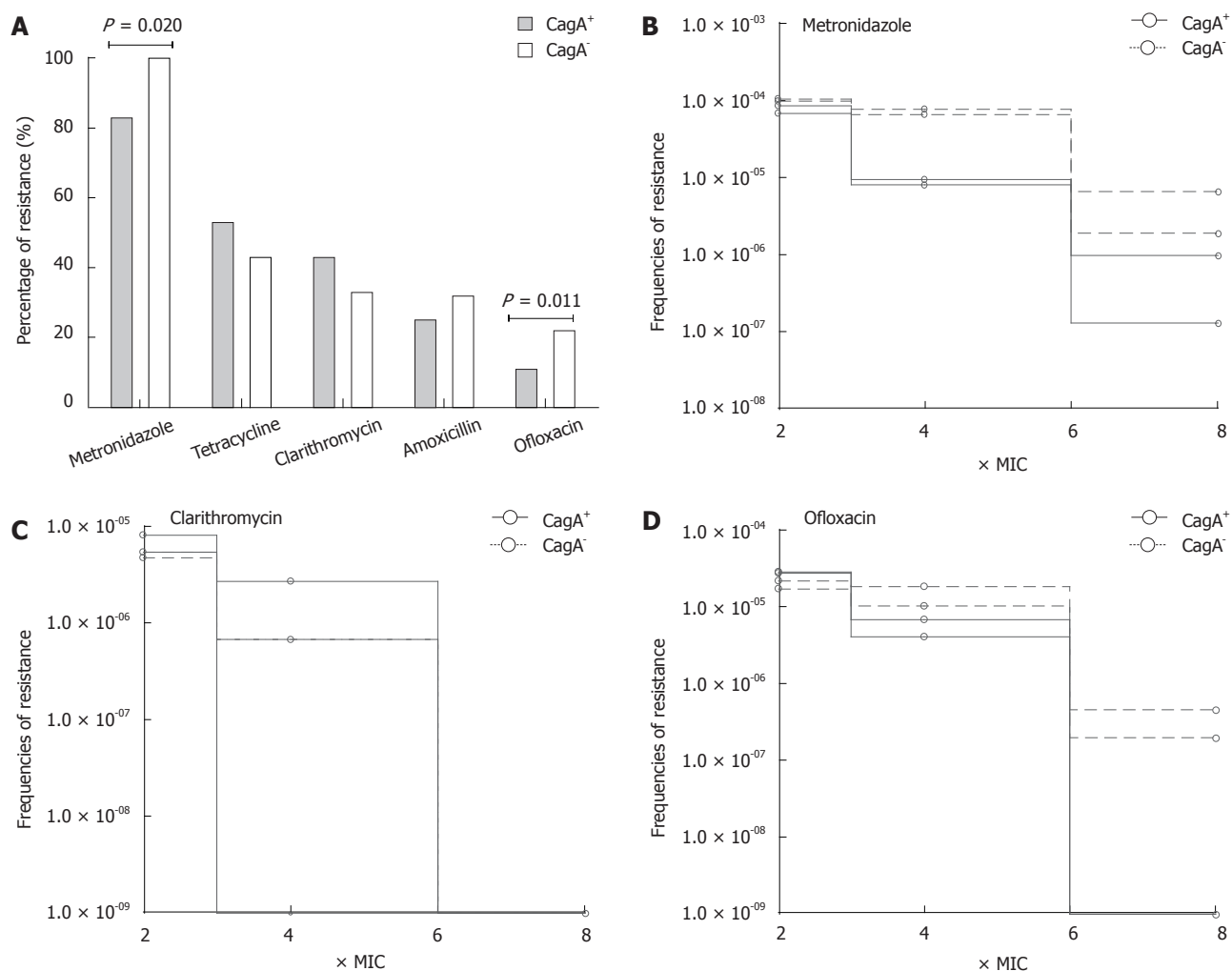


Figure 4 The correlation of antimicrobial drug resistance with *cagA* gene. A: Rate of drug resistance in *Helicobacter pylori* (*H. pylori*) strains carrying ($n = 81$) and devoid of ($n = 72$) of *cagA* gene. Statistical differences were observed by Pearson's χ^2 test. To determine the effect of *cagA* carriage on the development of resistance, *cagA*⁺ ($n = 2$) and *cagA*⁻ ($n = 2$) *H. pylori* strains were exposed to the increasing concentrations of metronidazole (B) clarithromycin (C) and ofloxacin (D). Bacterial growth was monitored at different concentration of antibiotic and frequencies of resistant mutants were determined as the colony forming units of *H. pylori* strain divided by the starting inocula.

cases. Of these, 81 (53%) were *cagA*⁺ and 72 (47%) were *cagA*⁻. Analysis of the drug resistance indicates the lower prevalence of OFX (11%, *n* = 9), MTZ (83%, *n* = 67) and AML (25%, *n* = 20) resistance in *cagA*⁺ strains compared with 22% (*n* = 16), 100% (*n* = 72) and 32% (*n* = 23) in *cagA*⁻ strains respectively (Figure 4A). In contrast, CLR resistance was more prevalent in *cagA*⁺ strains (43%, *n* = 35) than in *cagA*⁻ (33%, *n* = 24). The analysis indicates a possible link between *cagA* gene and the development of drug resistance.

To determine whether the rate of acquisition of antibiotic resistance varies between *cagA*⁺ and *cagA*⁻ strains, we exposed selected strains to increasing concentrations of MTZ, OFX and CLR. Bacterial growth was monitored at each concentration of antibiotics and frequency of resistant mutants was determined as the CFU of *H. pylori* strain divided by the starting inocula. We observed that *cagA*⁻ strains were able to mutate more frequently under the selective pressure of MTZ since they were able maintain their frequencies even after the exposure of $6 \times \text{MIC}$ of MTZ (r^2 0.9966, *P* = 0.0374). In contrast, more than 1 log₁₀ decrease in bacterial growth was observed in *cagA*⁺ strains with increasing concentrations of MTZ (Figure 4B). Similarly *cagA*⁻ strains maintained their frequency in the increasing concentration of OFX (r^2 = 0.9966, *P* = 0.0374) whereas a sharp decline was observed in the development of resistant mutants of *cagA*⁺ strains (Figure 4D). However, no significant difference was observed in the case of clarithromycin (Figure 4C).

DISCUSSION

H. pylori is often neglected for antimicrobial susceptibility testing because of its complex growth requirement and low recovery rate by bacterial culture. Increasing reports of treatment failure necessitate surveillance studies to analyze the trend of drug resistance especially in developing countries where MDR is quite common in other bacterial species. To determine the trend of antibiotic resistance in Pakistan, we conducted a 4-year longitudinal study comprised of 178 *H. pylori* strains. AST profile revealed high levels of resistance against the first-line regime including MTZ (84%), CLR (36%) and AML (37%). Our results and those from recently published papers from other countries show comparable prevalence rates; for example 33% resistance to AML was observed in the United States^[17] whereas MTZ and CLR resistance rates were 31% and 33% in Ireland^[18], 61% and 26% in France^[19], 48% and 28% in Saudi Arabia^[20], 80% and 45% in India^[21], and 77% and 15% in Bangladesh respectively^[22]. Moreover the trends of CLR and MTZ resistance were also in agreement with previously published reports^[6,23]. In contrast with our observations, available data indicate a low occurrence of such strains in southeast Asian countries such as Malaysia and Taiwan where *H. pylori* is endemic^[6,24]. However, the distinct genotypic nature of Southeast Asian strains provides a possible explanation for the differences in resistance profiles compared with rest of the world, including Pakistan.

The global analysis of clinical data clearly indicates that drug resistance to AML, MTZ and CLR has a central role in poor patient compliance to “gold standard” triple therapy to *H. pylori* infection, especially in the case of CLR if the point mutations in peptidyltransferase of 23S *rRNA* gene are responsible to phenotypic behavior^[25]. Therefore, Maastricht III consensus guidelines proposed not to provide CLR based empirical therapy if primary resistance rates are more than 15%-20% in the respective territory^[4]. The present study clearly indicates the upward trend in the primary resistance to CLR in our population with an average of 36% in mono-resistance and 22% in MDR (*R-phenotype*; MTZ^rCLR^rAML^r) strains which provides an possible reason for the poor patient compliance (up to 70%-75%) with CLR based therapies in Pakistan as reported earlier.^[8,9] Although MDR strains were equally present in our studied population when compared with the rates in other countries^[26,27], they were less prevalent than mono-resistant strains.

Fluoroquinolones such as ofloxacin or ciprofloxacin and tetracycline are usually considered as second-line therapy for *H. pylori* infection. In this study, the prevalence of TE resistance was comparable to that of other countries, however resistance to OFX was at a higher level than that seen other countries^[18,28]. Mutations in the *gyrA* gene that are responsible for fluoroquinolone resistance have been directly linked with the failure of *H. pylori* eradication^[29] therefore the higher rate of OFX resistance is alarming. These antibiotics are generally used to treat gastrointestinal infections in Pakistan; consequently, the resistance occurs in other Gram negative bacteria such as *Salmonella*, *Shigella* and *Escherichia coli*^[30] and therefore the transmission of resistance in *H. pylori* can be anticipated. To combat the situation, broad-spectrum fluoroquinolones such as levofloxacin have been introduced; however, the development of resistance and intense side effects hamper its wide use despite its better compliance rate^[31].

Genotypic differences of *H. pylori* directly influence the pathogenesis of infection. Such effects have been widely evidenced with the *cagA* gene carriage however the exact mechanism remains elusive. In this study, the differential prevalence of MTZ and OFX resistance in *cagA*⁺ and *cagA*⁻ strains clearly indicate the absence of *cagA* gene contributes in the acquisition of resistance which was further evidenced by the differential frequencies of resistant mutants developed with the increasing amount of each antibiotic as previously observed by Taneike *et al*^[16] previously. The underlying phenomenon is usually explained by the ability of *cagA*⁺ strains to cause intense inflammation which might increase the availability of antibiotics at the site of infection and eventually lead to better eradication of infection. In other words, it describes no direct role of *cagA* gene in antibiotic resistance. However, undermining the hypothesis, we observed that drug sensitive strains were more prevalent in NUD cases, despite of the absence of *cagA* gene, compared to those patients with damaged gastric mucosa. Taken together, the present study suggests that *cagA* gene and the degree

of tissue damage might be two independent factors that affect the drug susceptibility of *H. pylori*.

In summary, we observed that the magnitude of drug resistance in *H. pylori* strains is alarming in Pakistan. The degrees of gastric inflammation and bacterial genotypes are independently implicated in the development of resistance. The study reaffirms the need for both the continuous surveillance for drug resistance and the development of effective prevention and treatment strategies at national and regional levels.

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COMMENTS

Background

Eradication of *Helicobacter pylori* (*H. pylori*) is directly associated with symptomatic relief in the patients of gastroduodenal diseases. However, the failure of combination therapy containing two antibiotics and a proton pump inhibitor often results because of antibiotic resistance.

Research frontiers

Pattern of antibiotic resistance in *H. pylori* varies in different settings. However it is yet to determine that how host and pathogenic factors affect the prevalence of resistance.

Innovations and breakthroughs

This study indicates the alarming level of antibiotic resistance among Pakistani strains of *H. pylori* especially the magnitude of clarithromycin resistance is on rise and more commonly observed in non ulcerative dyspeptic patients. We further describe that *cagA* gene carriage and the degree of gastric inflammation are two independent factors affecting the metronidazole and ofloxacin resistance in *H. pylori*.

Applications

It is important to conduct continuous surveillance of antibiotic resistance in *H. pylori*. This study helps to comprehend antibiotic resistance pattern in *H. pylori* that facilitate to developing effective treatment strategy in different groups of patients.

Terminology

Non ulcerative dyspepsia (NUD) is defined as presence of upper gastrointestinal tract symptoms such as stomachache, indigestion and vomiting in patients who did not have damaged gastric mucosa.

Peer review

Overall, the study was well carried out and generally well written. However there are a few areas that needs further clarification mainly in the results section.

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