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High frequency of metronidazole and clarithromycin-resistant *Helicobacter pylori* in formalin-fixed, paraffin-embedded gastric biopsies

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ABSTRACT

Background: Clarithromycin and metronidazole resistance of *Helicobacter pylori* is increasing worldwide and has resulted in a loss in the effectiveness of therapeutic regimens. We aimed to evaluate common mutations of resistance genes to clarithromycin (A2143G, A2142G and A2142C) and metronidazole (*rdxA* and *frxA*) in *H. pylori* strains in formalin-fixed, paraffin-embedded gastric biopsies.

Methods: A total of 110 tissue blocks from children suspected of *H. pylori* infection were included. After DNA extraction, *UreC* PCR was performed. Specific primers and restriction enzymes by PCR-RFLP were used for analysis of A2143G and A2142G mutations. To detect A2142C and assess frequent mutations of metronidazole resistance, specific primers and PCR method were used.

Results: One hundred cases of *H. pylori* (91%) were by PCR. Of 34 (34%) clarithromycin-resistant isolates 17 (50%), 10 (29%) and 7 (21%) had A2143G, A2142G, A2142C, respectively. Resistance rate to metronidazole was 60% ($N = 60$). In sequencing *rdxA* and *frxA* in the mutated strains, missense mutations were most frequent (60 and 57%, respectively), and there were differences in frameshift and non-sense mutations ($p < 0.001$).

Conclusion: Resistance rate to clarithromycin was high and the highest percentage of mutation was of A2143G. PCR-RFLP was used directly with formalin-fixed gastric biopsies, thus, avoiding the requirement for time-consuming culture-based methods. The isolates that developed resistance were mainly associated with mutations of both *rdxA* and *frxA* genes.

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Introduction

Helicobacter pylori infection is a causal factor in the pathogenesis of chronic gastritis [1], peptic ulcer [2] and gastric cancer [3], considered as one of the most genetically various bacterial species [4] and in one study was present in more than 50% of children infected before the age of 15 [5]. Effective antibiotic therapy often eradicates bacteria and results in the resolution of disease [6]. Various regimens with combination of different antimicrobials and acid-suppressing agents have been used with variable success. Among them, treatment regimens of a proton pump inhibitor and combination of two or more antibiotics (metronidazole, clarithromycin, amoxicillin or tetracycline) are considered most effective [3]. However, resistance to clarithromycin and metronidazole has become more frequent and is now likely to be the main cause of treatment failure of *H. pylori* eradication therapy [7].

Molecular genetics methods have an expanding role in the rapid detection of resistance to the mentioned antibiotics, determination of the latter hampered by limitations of bacterial culture and low sensitivity [8]. Furthermore, the probability of evaluating mutate clarithromycin-resistant genotypes by PCR on archived paraffin-embedded biopsy specimens has further emphasized the efficacy of these methods [7,9].

Resistance to clarithromycin is caused by the point mutations in the peptidyl transferase-encoding region of 23S *rRNA* which affects the binding of macrolides to the bacterial ribosome [10]. Three main 23S rRNA point mutations (A2143G, A2142G, A2142C) have been shown to be responsible for >90% of clarithromycin resistance cases, and different prevalence rates of such mutations have been reported [7].

The first demonstration of major mechanism of metronidazole resistance in *H. pylori* [11] showed that it was due to null mutations in the *rdxA* gene, which encodes an oxygen-insensitive NADPH nitroreductase. An additional putative metronidazole nitroreductase-encoding gene (NADPH flavin oxidoreductase; *frxA*) was similarly reported to involve metronidazole resistance. Simultaneous alterations in *rdxA* and *frxA* genes may induce a higher level of metronidazole resistance [6].

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The purpose of our study was to detect *H. pylori* isolates in paraffin-embedded tissues and evaluate the common mutations of resistance genes to clarithromycin (A2143G, A2142G and A2142C) and metronidazole (*rdxA* and *frxA*) in *H. pylori*.

Materials and methods

Analysis was performed on 110 formalin-fixed, paraffin-embedded tissue blocks from children (aged up to 15 years) attending Children's Medical Centre Hospital, Tehran, Iran for upper gastrointestinal endoscopy of suspected *H. pylori* infection. Samples were isolated from gastric biopsies obtained during upper gastroduodenal endoscopy. Fifty-three patients were female (53%) and 47 patients were male (47%), with a median age of 6 years (IQR: 4 year and 2 months–8 years).

Deparaffinization from paraffin-embedded blocks was performed as described previously [12]. Briefly explaining, two 10 µm sections cut from each tissue block were deparaffinized by adding 1 ml xylene and incubation at 37 °C for 10 min, then centrifuged for 5 min. The xylene was removed with 95% ethanol. The tissue pellets were treated with 100, 90, 80 and 70% ethanol. For DNA extraction, we selected a commercially available DNA extraction kit (TIANquick FFPE DNA Kit, TIANGEN Biotech) and performed the procedures according to the manufacturers' guidelines.

In order to confirm whether samples were positive for *H. pylori*, PCR for *UreC*, a highly conserved and very sensitive and specific for the detection of *H. pylori* [13], was performed using the primers and cycling programme shown in Table 1 [10]. A 10 ul sample of PCR-amplified products was electrophoresed on a 1% gel, was stained with GelRed (Biotium), and examined under UV light for the presence of the 249 bp fragment of amplified DNA.

Primers Cla18 (5′-AGTCGGGACCTAAGGCGAG-3′) and Cla21 (5′- TTCCCGCTTAGATGCTTTCA-3′) were used to determine A2142G and A2143G mutations by a PCR-RFLP method and digestion with *MboII* (Thermo Fisher Scientific) and *BsaI* (Thermo Fisher Scientific) which the results demonstrated to be one of three types of the wild, A2142G mutant (*MboII* restriction site), and the A2143G mutant (*BsaI* restriction site) [14]. The restriction products were analysed by electrophoresis on a 1% agarose gel. Presence of A2142C mutation was detected using specific primers Cla3 (5′-AGGTCCACGGGGTCTTG-3′) and Cla18, as described previously [14]. Also, a 700-bp fragment was detected with a 1.5% agarose gel, providing the 23S rRNA gene had the A2142C mutation [10].

In addition, PCR for *rdxA* and *frxA* was performed in order for evaluation of metronidazole resistance, using their specific primers. The details are illustrated in Table 1. Statistical data analysis was performed using the SPSS software version 18.0. Differences in missense, frameshift and non-sense mutations in *rdxA* and *frxA* were determined by chi-squared testing.

Results

Of the 110 paraffin-embedded samples examined, 100 (91%) were positive by PCR for *UreC*. Of 100 *H. pylori* isolates, 34% ($N = 34$) showed clarithromycin resistance. To study common mutations of resistance genes to clarithromycin, a 1.4 kb fragment of the 23S rRNA gene was amplified in all isolates (Figure 1(a)), followed by *MboII* and *BsaI* digestion (Figure 1(b) and (c)). All RFLP analysis indicated that 27 (27%) of isolates had mutations, among which 63% had the A2143G mutation and 37% had the A2142G mutations. In addition, 7 (7%) of isolates with a A2142C mutation had this mutation in their 23S rRNA gene.

Resistance rate to metronidazole was 60% ($n = 60$). Missense, frameshift and non-sense mutations were found in 36 (60%), 10 (17%) and 14 (23%) of *rdxA* analyses, and in 34 (57%), 24 (40%) and 2 (3%) of *frxA* analyses, respectively ($p < 0.001$). In both analyses, missense mutations were most frequent. The frequency of frameshift mutations in *frx*A was higher, but that of non-sense mutations was lower than in *rdx*A ($p < 0.001$).

Discussion

H. pylori infection is a common chronic gastric infection all around the world. Although eradication therapy of *H. pylori* has improved the clinical outcome of patients with various upper gastrointestinal diseases [15], the antibiotic resistance of *H. pylori* is increasing worldwide and has resulted in a loss in the effectiveness of the current therapeutic regimens [16]. Resistance to clarithromycin as the most powerful antibiotic currently used for *H. pylori* treatment is suggested as the main predictor of failure of eradication. Because of an increased usage of these macrolides, the prevalence rate of resistant strains are increasing and the detection of resistance is becoming of major importance [14].

Table 1. Primer sets and PCR protocols used in this study to amplify UreC and evaluate metronidazole resistance.

Primer	Nucleotide sequence	Product size	PCR condition
UreC-F	AAGCTTTTAGGGGTGTTAGGGGTTT	249	35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min
UreC-R	AAGCTTATTTCTAACGC		
rdxA-F	GCAGGAGCATCAGATAGTTC	880	30 cycles of 94 °C for 40 s, 50 °C for 40 s, and 72 °C for 1 min
rdxA-R	GGGTGATTTCTTGGTTGC		
frxA-F	GAGAACAAGTGATTGCTTTA	654	30 cycles of 94 °C for 40 s, 54 °C for 40 s, and 72 °C for 1 min
frxA-R	GCAGGAGCATCAGATAGTTC		

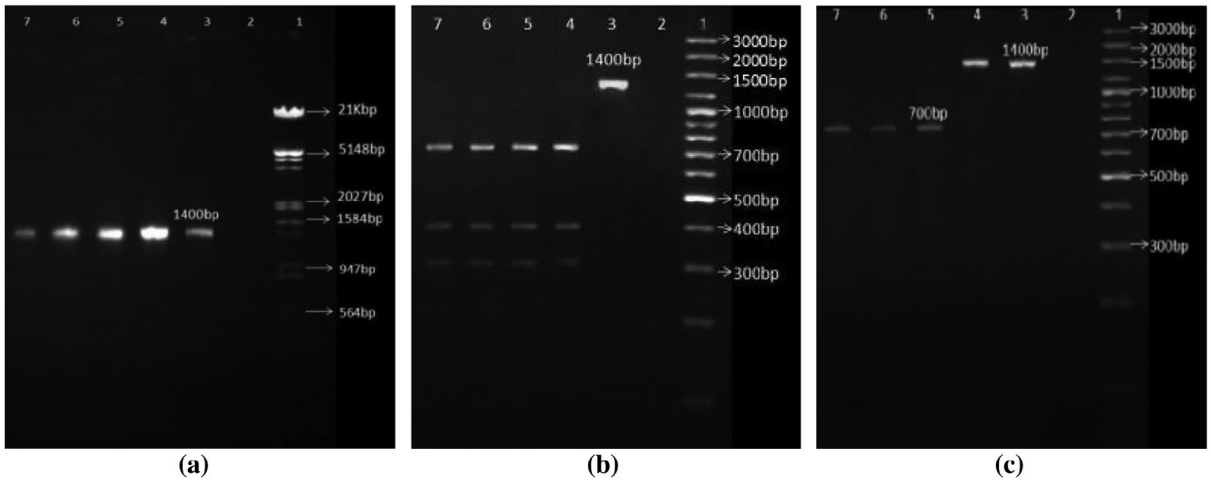

Figure 1. Gel electrophoresis of PCR products to detect A2143G and A2143G mutations. (A) PCR product of 1.4 kb fragment of 23S rRNA gene, lane1: Ladder (B) *BsaI* digestion producing, 700, 400 and 300 bp product that shows A2143G point mutations, lane1: Ladder, lane4–7: the strains with A2143G mutation. (C) *MboII* digestion producing 700 bp fragment that shows A2142G point mutations, lane1: Ladder, lane5–7: the strains with A2142G mutation.

In our study, 91% of samples were positive for *H. pylori*, a rate comparable to the figures reported elsewhere (77–100%) [17,18]. According to the results a systematic review, prevalence of clarithromycin resistance in our population was 22% [19], but in the current study resistance rate to clarithromycin was 34%, much higher than the resistance rate reported elsewhere of 9.5–15% [20–22]. A decade ago, the prevalence of resistance in *H. pylori* among Iranian children was very high for metronidazole (95%) and amoxicillin (59%), moderate to clarithromycin (16%), and low to ciprofloxacin (7%) and tetracycline (5%) [23].

Among clarithromycin-resistant isolates, A2143G, A2142G and A2142C mutations were detected in 17, 10 and 7%, respectively. This contrasts with a report from Tunisia, where the rate of these mutations was 88.1, 11.9, and 0%, respectively [24]. The frequency of two first mutations were higher than ours, while the overall rate of resistance to clarithromycin was lower (18.8%) [24]. In a study of 106 *H. pylori* isolates, four had A2143G (75%) or A2142G (25%) mutations [25]. In our previous study [26], the A2143G point mutation was detected in 71% of clarithromycin-resistant strains which was marginally higher than the current study.

Analysis for clarithromycin resistance by Kargar et al. [14] showed a noticeable difference between two Real-time PCR and PCR-RFLP, with of 36 and 24.67%, respectively. Therefore, it is possible that the different methods used may have played a role in the discordant data [7]. The presence of multiple strains identified by real-time PCR may be missed by the PCR-RFLP method [14].

Metronidazole has been chosen as another antibiotic for treatment of *H. pylori*, achieving a satisfactory eradication rate [27]. However, higher resistance to metronidazole is higher in developing countries (20–30%) compared to Europe (10%) and is attributed to the frequent use of metronidazole to treat other conditions [28,29]. In the current study, the resistance rate to metronidazole was 60%, higher than the rate reported by Alfizah et al. (36.6%) [30].

Several studies of fresh clinical isolates indicated that metronidazole resistance are due to a point mutation in *rdxA* (HP0954, in the fully sequenced genome of strain 26695) [31]. In the study conducted in Taiwan in high-level metronidazole-resistant (MIC ≥ 64 μg/mL) *H. pylori* isolates, 89% of metronidazole-resistant isolates had mutation of rdxA [6]. In a small study, of 18 isolates infected by *H. pylori*, 14 (78%) had mutations in *rdxA* and *frxA*. One isolate had *frxA* mutation without any change in *rdxA* gene and there was no alteration in both in one isolate, showing that other factors contribute to resistance to metronidazole [32]. In our study, missense mutations in *rdxA* and *frxA* were the most common mutation, compatible with those of others [30]. Another small study (from the USA) reported that six of 12 resistant *H. pylori* isolates had a non-sense mutation, while two isolates had a missense mutation and seven isolates had frameshift mutation in *rdxA* [33]. These are markedly different to our data and may be due to local conditions, methods, organism strain, sample size bias and the nature of the patients and their co-morbidities. The resistance rate to the both evaluated antibiotics in the current study might have so many effects on efficiency of treatment regimens. High prevalence of clarithromycin or metronidazole-resistant strains can lead to more variety of mutant strains and the incidence of secondary resistance to these antibiotics. Determination of resistance to these antibiotics before prescribing treatment regimens is vital to prevent from a secondary resistance to them and imposition of additional costs of treatment.

In conclusion, resistance rate to clarithromycin was high and the highest percentage of mutations were of A2143G. As PCR-RFLP was used directly with formalin-fixed gastric biopsies, the requirement for

time-consuming culture-based methods was avoided. This work represents an advance in biomedical science because determination of resistance to clarithromycin and metronidazole before prescribing treatment regimens is vital to prevent secondary resistance and additional costs of treatment.

Summary table

What is known about this subject:

- *H. pylori* infection is a common chronic gastric infection all around the world.
- The antibiotic resistance of H. pylori is increasing worldwide and has resulted in a loss in the effectiveness of the current therapeutic regimens

What this paper adds:

- Among clarithromycin-resistant isolates, A2143G, A2142G and A2142C mutations were detected in 17, 10 and 7%, respectively.
- Sequencing *rdxA* and *frxA* showed that among the mutated strains, missense mutations were the most frequency.
- The frequency of frameshift mutations in *frxA* is higher, but that of non-sense mutations is lower, than in *rdxA*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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