Original article

A study of *rdxA* gene deletion in metronidazole resistant and sensitive *Helicobacter pylori* isolates in Kerman, Iran

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Abstract
Introduction and objective: Metronidazole (Mtz) resistance in *Helicobacter pylori* has been found to be associated with mutations in *rdxA*, a gene encoding an oxygen insensitive NADPH nitroreductase, and enhanced by mutations in *frxA* a gene encoding a NAD(P)H-flavin oxidoreductase. In this study, we examined the prevalence of *rdxA* deletion among local isolates of *H. pylori*.
Materials and methods: We tested 63 *H. pylori* isolates obtained from 191 patients referred to endoscopy unit of Afzalipour hospital in Kerman, Iran, during 2009 for their susceptibility to common anti-*H. pylori* antibiotics using a modified disk diffusion test. Then the metronidazole resistant and sensitive isolates were evaluated for deletion in *rdxA* gene by PCR methods and compared with each other.
Results: From 35 resistant *H. pylori* isolates in this study only 22.9% (8 isolates) detected with deletion *rdxA* gene. No *rdxA* deletion was detected in sensitive isolates.
Conclusion: According to *rdxA* deletion rates in this study, it seems that some other nitroreductases are involved in metronidazole activation or there are other metronidazole resistance mechanisms involved.

Keywords: *Helicobacter pylori*; Metronidazole resistant; *rdxA* deletion

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Introduction

Helicobacter pylori is a Gram-negative, microaerophilic spiral bacterium that colonizes the human gastric mucosa of about 50% of the world's population [1]. The outcomes of this infection are gastric inflammation, peptic ulcer, gastric ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [2]. Commonly prescribed therapies are based on the combination of a proton pump inhibitor with two antibiotics: metronidazole with clarithromycin or amoxicillin. Resistance to metronidazole, clarithromycin and/or is the main reason of the failure of such regimens [3].

Metronidazole (Mtz) is a nitroimidazole used principally for the treatment of anaerobic and parasitic infections. Mtz is stable at a low pH and is actively secreted into the gastric juice. The chemical formula of this antibiotic is 2-(2- methyl- 5-nitro- 1*H*- imidazol- 1-yl) ethanol and its bioavalability is 100% oral and 59%-94% injectional. The half life of this antibiotic is 6-7h [4].

Metronidazole is administered as a prodrug that is activated by the reduction of the nitro group that is attached to an imidazole ring. Since oxygen has a higher redox potential than metronidazole, this reduction step works out most effectively in an environment with low oxygen tension, such as anaerobic cells and protozoa. Surprisingly, the drug was also found to be active against the microaerophilic pathogen *H. pylori* [5]. Metronidazole resistance is common in *H. pylori*, and can be variable from 10% in developed countries to 50% in developing countries [6].

For the first time Goodwin *et al.* [7] announced that inactivation of an oxygen-insensitive NADPH nitroreductase (*rdxA*) may be responsible for metronidazole resistance. Metronidazole must be reduced in order to be active, and *H. pylori's* redox systems with a potential lower than that of Mtz (-415 mV) would be capable of donating electrons to Mtz. Reduction products have been demonstrated to cause breakages in DNA which results in cell death [8].

The activation of metronidazole in strictly anaerobic bacteria is mediated by the pyruvate: ferredoxin oxidoreductase complex [9]. For example, this function in *H. pylori* might be fulfilled by the electron carriers, *RdxA* (HP0954), *FrxA* (HP0642), ferredoxin (FdxA, HP0277), flavodoxin (*FldA*, HP1161), pyruvate: ferredoxin oxidoreductase (*PorD*, HP1109) and 2-oxoglutarate ferredoxin oxidoreductase (*OorD*, HP0588).

Inactivation of the genes involved in some of these systems has been found to be linked to Mtz resistance [5]. Mtz resistance pattern of *H. pylori* and its' molecular mechanisms are geographically different. Therefore, a need for local studies is felt. The aim of this study was to investigate the *rdxA* gene deletion in both Mtz resistant and sensitive *H. pylori* isolates from Kerman (south east of Iran).

Materials and methods

Bacteria

Sixty three *H. pylori* isolates were obtained from 191 patients biopsy samples referred to the endoscopy unit of Afzalipour hospital in Kerman, Iran, during 2009. The biopsy samples were cultivated in brucella agar medium (Merck, Germany), supplemented with 10% defibrinated sheep blood and three antibiotics (vancomycin 10mg/l amphotericin B 10mg/l and trimetoprim 5mg/l). The inoculated plates were incubated at 37°C under microaerophilic atmosphere provided by anerocult C (Merck, Germany) for 3-5 days. The isolates were recognized as *H. pylori* by urease, catalase, oxidase positive and Gram-negative staining tests [10].

Antibiotic susceptibility tests

The susceptibility of the isolates to metronidazole was evaluated by disc diffusion method. However there is no standard method to evaluate the susceptibility of *H. pylori* to antibiotics; we used the NCCLs recommended method called modified disc diffusion method. In this method, we prepared a microbial suspension equal to 4 McFarland turbidity (12×10^8CFU/ml) and cultivated in Muller-Hinton agar (Merck, Germany) supplemented with 10% defibrinated sheep blood. The 5µg metronidazole disc (Mast, England) was placed in the plates and incubated in 37°C under microaerophilic atmosphere for three days. The inhibition zone of ≥ 21mm was considered susceptible and the inhibition zones less than that were considered as resistant [10-11].

DNA extraction and amplification

DNA was extracted from all 63 *H. pylori* isolates by genomics kit (Bioneer, South Korea) according to the manufacturer's instruction. The expected fragment was 850bp if the gene was normal but 650bp if the gene was mutated. PCR condition was as follows: reactions were carried out in MWG thermo cycler in 25µl mixtures containing 12.5µl PCR master mix (Cinna gen, Iran), 9.5µl sterile deionized water, 1µl template DNA and 1µl of each oligonucleotide primers. Initial denaturation at 94°C for 5mins followed by 30 cycles of denaturation at 94°C for 1min, annealing for 1min at 55°C, extension at 72°C for 1min. The final extension step was extended to 10min at 72°C (Table 1).

Table1: Primers used for amplifications

Primer name	5'-3' sequence	Expected fragment	Reference
rdxA1	AATTTGAGCATGGGGCAGA	850bp	12
rdxA2	GAAACGCTTGAAAACACCCCT		

Electrophoresis

The PCR products were separated on 1.5% agarose gels (Cinna gen, Iran) in TBE 1X (Tris/borate/EDTA) buffer. Bands were visualized under UV gel documentation and photographed. Ethidium bromide (Merck, Germany) as a stain has been added to the agarose gel during preparation to give a concentration of 0.2µl/ml.

Statistical analysis

Data were analyzed by SPSS version 16.0. The Pearson chi-square test was used to assess the relationships between the results of disc diffusion and PCR methods.

Results

From 191 patients referred to endoscopy unit of Afzalipour hospital in Kerman, Iran, 57.6% (110 cases) were female and 42.4% (81 cases) were males. This population was grouped into four categories: 1) under 30 years old, 2) 30-40 years old, 3) 41-50 years old, 4) more than 50 years old. The *H. pylori* isolates obtained from 25 males and 38 females (63 *H. pylori* isolates totally). 55.5% of isolates (35 out of 63 isolates) were resistant to metronidazole. There was no significant relation between gender and metronodazole resistant but there was a significant relation between age of patients and metronidazole resistant.

The rate of metronidazole resistance in the isolates from 30-40 year old patients was significantly higher than that of other age groups (p=0.037). There was a significant correlation between deletion of *rdxA* gene and metronidazole resistance. 22.9% of the metronidazole resistant isolates (8 isolates) had 200bp deletion in *rdxA* gene while none of the sensitive isolates had this mutation (Table 2 & Fig. 1). There was no significant correlation between gender or age and *rdxA* deletion.

Table 2: Metronidazole resistance patterns of *H. pylori* isolates and evaluation of *rdxA* gene deletion in both resistance and sensitive isolates in Kerman, Iran

Mtz* susceptibility situation	No (%)	*RdxA* deletion	No (%)
R†	35 (55.5%)	R	8 (22.9%)
Sσ	28 (45.5%)	S	0 (0.0%)

*Mtz: Metronidazole, R†: Resistant, Sσ: sensitive

Fig. 1: Agarose gel electrophoresis of PCR products of *rdxA* gene. No 1: 1000bp DNA ladder, No 2: negative control, No 3, 6 & 9: rdxA gene deletion negative (phenotype: Mtz sensitive), No 4, 5, 7 & 8: rdxA gene deletion positive (phenotype: Mtz resistant)

Discussion

The metronidazole resistance is a worldwide concern [12]. It seems that the abundant usage of metronidazole in both *H. pylori* and non *H. pylori* infections such as parasitic infections is a main reason for emergence of metronodazole resistance. In fact, the antibiotic acts as an external agent that selects the resistant strains [13]. The highest rate of metronidazole resistance was seen among isolates from 30-40 year old patients. This could perhaps be due to the high rate of infection in this age group according to Malekzadeh *et al.* [14].

The molecular mechanisms of Mtz resistance are now recognized. Goodwin *et al.* [7] announced that inactivation of an oxygen-insensitive NADPH nitroreductase (*rdxA*) may be responsible for metronidazole resistance. One of these inactivation mechanisms is a deletion in *rdxA* gene that we studied here [15]. However we noticed a significant correlation between *rdxA* gene deletion and

metronidazole resistance, but only 22.9% of the isolates (8 out of 35) showed the *rdxA* gene deletion.

One of the other mechanisms that may be associated with *rdxA* gene inactivation is insertion of a transposon called Mini-IS605 [12]. In *H. pylori*, metronidazole resistance is primarily associated with mutational inactivation of the *rdxA* gene. Recently it has been demonstrated that inactivation of the *frxA* gene also confers metronidazole resistance, either alone or in association with the *rdxA* gene [16,17]. Whether mutational inactivation of these two enzymes accounts for metronidazole resistance in all clinical isolates is still being debated, but, they, most likely reflect the two major contributing factors [18].

Efflux pumps are actually some of the cell membrane proteins that efflux the antibiotics to the external space of the cell and don't permit the antibiotics to be accumulated to toxic levels. The recent studies showed, presence of an efflux pump named "TolC" is associated with resistance to metronidazole [19].

Conclusion
We showed that *rdxA* gene deletion is associated with metronidazole resistance, but this is not the only mechanism of such a resistance and there may be some other mechanisms involved. We suggest further investigations to be carried out for other probable mechanisms of metronidazole resistance like *frxA* gene inactivation, insertion of Mini-IS605 and presence of efflux pumps.

Acknowledgment

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