Role of NADPH-insensitive nitroreductase gene to metronidazole resistance of Helicobacter pylori strains

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ABSTRACT

Background and the purpose of the study: Current anti-H. pylori therapies are based on the use of two antibiotics with a proton pump inhibitor and/or a bismuth component. Metronidazole is a key component of such combination therapies in Iran. The aim of this study was to determine the role of rdxA gene in resistant strains of H. pylori isolated from Shahrekord Hajar hospital to metronidazole.

Methods: This study was a cross-sectional method, which was carried out on 263 patients who referred to endoscopy department of Hajar hospital, in 2007. Biopsy samples were cultured on selective Brucella agar containing 10% blood and incubated under microaerophilic condition at 37°C for 3 - 7 days. Suspected colonies were tested by Gram staining, urease, oxidase and catalase activities. Organisms were confirmed to be H. pylori on the basis of the presence of ureC (glmM) gene by PCR. Specific primers were used for detection of rdxA gene mutation.

Results: Eighty and four strains of H. pylori determined by PCR method. Of the isolated strains, 49 (58.33%) were resistant, 7 (8.33%) were semi-sensitive to metronidazole and 200bp deletion in rdxA gene was observed in 2 strains.

Conclusion: Because of the high metronidazole resistance in patients under study it was necessary to replace it by other antibiotics in therapeutic regimens. On the basis of low frequency of resistance mutation in rdxA gene, sequence analysis for identification of other mechanisms is suggested.

Keywords: Helicobacter pylori, Metronidazole resistance rdxA gene

INTRODUCTION

Helicobacter pylori is a spiral, gram negative bacterium that has been recognized as a causative factor in gastritis, duodenal and peptic ulcer, gastric adenocarcinoma and MALT lymphoma (1). Resistance of Helicobacter pylori to either clarithromycin or metronidazole (Mtz) has been associated with therapeutic failure and reduced eradication rates with multi-agent treatment regimens (2). Multiple nitroreductase are expressed by H. pylori and probably contribute to the reductive activation of Mtz (3). In susceptible protozoan and bacterial pathogens to nitroimidazole compounds, reduction of nitro group is considered essential for formation of reductive intermediates which are likely to mediate chromosomal DNA strand breakage with resultant cytotoxicity (4). Inactivation mutational of rdxA gene, which encoding an oxygen-insensitive (type I) NADPH nitroreductase, confer Mtz resistance on H. pylori. It has been considered that rdxA gene is the primary nitroreductase responsible for reduction of the nitro group and activation of Mtz in H. pylori. Goodwin et al. demonstrated that insertion inactivation of rdxA in H. pylori resulted in a Mtz-resistance phenotype by preventing reduction of Mtz (3). The aim of this study was to determine the role of rdxA gene in metronidazole resistance of H. pylori strains isolated from Shahrekord Hajar Hospital in Iran.

MATERIAL AND METHODS

Patients
Totally 263 consecutive patients with dyspeptic symptoms attending the endoscopy suite of gastroenterology section of hospital of shahrekord university of medical sciences from July to December 2007 were enrolled. Each patient’s history sheet was examined in detail and findings were recorded on standard performa including demographic data. Those with positive history for above drugs were excluded. All patients read and signed an ‘informed consent’ form at the beginning of endoscopy and declared their willingness for the application of
out of the 84 strains (between patient gender and prevalence of metronidazole-resistant susceptibilities of metronidazole resistant (Fig 2). The results showed no correlation between the metronidazole

![Figure 1. PCR products of ureC gene. Lane 1 - 4 294bp fragments, lane 5 positive control, lane 6 negative control, M: DNA size marker.](image)

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their anonymous data for research purpose. For each patient, three biopsy specimens were taken, from the antrum, the gastric body, using a disinfected endoscope, were placed in 0.1 ml of sterile saline solution and sent to clinical microbiology laboratory of Islamic Azad University in Shahrekord.

**Bacteria and culture conditions**

Biopsy samples were cultured on Brucella agar (Merck) supplemented with 7% fresh horse blood, vancomycin (6mg/l) (Merck), trimethoprim (5mg/l) (Merck) and amphotericin (2mg/l) (Merck). For primary culture, plates were incubated at 37°C in a microaerophilic atmosphere (5% O2, 15% CO2, 80% N2), for 3 - 5 days. Strains were identified according to colony morphology, Gram stain and positive reaction with urease, catalase, oxidase. The ureC (glmM) which encodes urease was used as a target DNA to confirm *H. pylori* strains.

**Antimicrobial susceptibility testing**

The susceptibilities of the *H. pylori* isolates were examined by an agar dilution method according to CLSI (Clinical and Laboratory Standard Institute) (5). Resistance breakpoint for metronidazole was defined as >8 µg/liter (5).

**DNA extraction and PCR assays**

The extraction of *H. pylori* genomic DNA was performed as reported previously (6). The ureC (glmM) gene was detected by using the primers 5’-AAGCTTTTAGGGTAGGGTTT-3’ and 5’-AAGCTTTAGGGTAGGGTTT-3’ with 35 cycle at 39 °C for 1 min, 55°C for 1 min, and 72°C for 1 min, which amplifies a 295-bp amplicon. PCR reaction was carried out in Gene Amp 9700 (Perkin Elmer) (6). Then seven microliter portions of the PCR products were analyzed by electrophoresis in 1.5% agarose gel using Tris-acetate-EDTA (TEA) buffer stained with ethidium bromide in parallel with a molecular weight marker: Gene ruler 100-bp DNA ladder (MBI Fermentase; Vilnius, Lithuania). For detection of metronidazole resistance, primers RdxA1 (5’-AATTTGAGCATGGGGCGA-3’) and RdxA2 (5’-GAAACGCTTGAACACCCCT-3’) were used for determination of deletion of *rdxA* gene. PCR amplification was performed in a thermal cycler (PE Applied Biosystems, Chiba, Japan), as described previously. The sizes of the PCR products of the *rdxA* gene were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide (0.5ml) (7, 8). The data were analyzed using SPSS software (SPSS for windows, 14 programs) and Chi-square and then Fisher’s exact tests. *P*-value less than 0.05 were taken to indicate statistical significance.

**RESULTS**

**Culture, RUT and PCR of biopsy specimens**

*H. pylori* was isolated from 84 of 263 (31.94%) patients participated in this study. Of these 35 (13.31%) were male patients, and 49 (18.63%) were female. The organism was successfully cultured from 55 out of 135 (40.74%) patients with non-ulcer dyspepsia and 29 out of 62 (46.77%) of patients with peptic ulcer. The percentage of culture positive specimens was 31.94 (84 out of 263) while a positive RUT and PCR results were observed in 54.37% (143 out of 263), 84.79% (223 out of 263) respectively (Figure 1).

**Prevalence of metronidazole resistance**

By an agar dilution method, out of the 84 *H. pylori* isolates, 49 (58.33%) were found to be metronidazole resistant (Figure 2). The results showed no correlation between the metronidazole susceptibilities of *H. pylori* isolates and patients age. There was a significant difference between patient gender and prevalence of metronidazole-resistant *H. pylori*. Out of 49 patients, 17 (20.24%) were male and 32 (38.09%) patients were female harbored resistant strains (*p* = 0). For 47
metronidazole resistance strains, the rdxA amplicon was approximately 800bp and for 2 resistance strains the rdxA amplicon was 600bp (Figure 3). There was no significant difference between rdxA gene deletion and Mtz resistance.

DISCUSSION

A major obstruction to successful H. pylori treatment is the presence of antibiotic resistant strains. The prevalence of H. pylori resistance to metronidazole varies from 20% to 40% in Europe and USA, with one exception in Northern Italy. It is well known that the prevalence is much higher in developing countries (50-80%), such as Mexico (76.3%) (9,10). In this study resistance to metronidazole was 58.33%, which was similar to resistance pattern in developing countries. Mtz resistance associated with mutations of rdxA gene is still one of the controversial topics. Firstly, Debets-Ossenkopp et al. showed that the 200bp deletion in rdxA gene was a major factor in MTZ resistance (8). In contrast, Kato et al. reported that there was no deletion of 200bp in rdxA gene in Mtz resistance strains (7). In accordance with these reports, in this study only in 2 (4%) strains, deletion of rdxA gene was identified. A troubling aspect of resistance to some antibiotics by H. pylori is a phenomenon that has been given the name heteroresistance. Unusually, in testing for resistance, only a single colony of the isolate under study is tested for its susceptibility to various antibiotics. Scientists have now found that if 10 colonies of a strain isolated from an ulcer patient are tested for resistance, they vary widely with respect to susceptibility. This raises the possibility that the appearance of resistance is simply due to selection of the resistant sub-population within the larger population of mostly susceptible bacteria. This phenomenon has been observed so far only with resistance to metronidazole, but it raises the troubling question of how much potential there is for strains of H. pylori to become resistant to antibiotics very rapidly (10).

In the present study resistance to metronidazole in women was higher than men, probably due to the use of niroimidazoles drugs to treat gynaecological infections. The rate of incidence of H. pylori infection in the developed countries may be as low as 30%, while in developing and under developing countries it is more than 80% (11). In this study, 84.79% of patients were infected; this is in agreement with report of Doosti et al. in 2006 in this region (12). However these rates vary wildly in different regions of Iran. For example rates of infection are 62.56%, 65.1% and 48% in Mashhad (13), Isfahan (14) and Semnan (15) respectively.

CONCLUSION

In summary, mutations in rdxA may not always be essential for metronidazole resistance. Future examination of rdxA expression at the transcription and translational level may provide further insight into the role of this locus in metronidazole action and resistance of H. pylori. On the other hand it seems that other mechanisms such as scavenging of toxic oxygen radicals by an altered catalase or superoxide dismutase is, a more efficient DNA damage repair mechanism, and loss of function of a critical reductase contributed to metronidazole resistance. Thus identification of other resistance mechanisms is suggested.

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