Original Article

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Susceptibility testing of *Helicobacter pylori*: Comparison of E-test and Disk Diffusion for Metronidazole and Mutations in rdxA gene sequences of *Helicobacter pylori* strains

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Abstract

Metronidazole is a main stay of modern multidrug therapies for *Helicobacter pylori* (*H. pylori*) infection. Metronidazole resistance reduces the effectiveness of these combination therapies. Various methods have been used for the determination of the sensitivity of *H. pylori* to metronidazole, that have shown conflicting results. The aims of this study are: 1) Comaring E-Test and disk diffusion methods for determining the susceptibility of *H. pylori* to metronidazole; and 2) As metronidazole resistance in *H. pylori* has been found to be associated with mutations in rdxA, the role of this gene in metronidazole resistance in *H. pylori* has been examined in this study. A total of 46 *H. pylori* strains from 223 consecutive patients were examined. The E-Test was performed according to the manufacturer's guidelines, and the disk diffusion test, according to standard procedure, using 5 µg metronidazole disks. Extraction of DNA was done from all *H. pylori* isolates by boiling and the use of phenol-chloroform methods, and afterwards Polymerase Chain Reaction (PCR) was performed. Metronidazole resistance as determined by E-test and disk diffusion methods, was 64.3% and 47.6% respectively. None of the resistant or sensitive samples possessed rdxA gene deletion. Disk diffusion method is not reliable in determining metronidazole resistance in *H. pylori*. An intact rdxA gene has also been reported in metronidazole-resistant *H. pylori*, suggesting that additional metronidazole resistance mechanisms exist in *H. pylori* and even molecular methods are not reliable for the detection of this resistance.

Keywords: Drug Resistance, *Helicobacter pylori*, Iran, Metronidazole, Polymerase Chain Reaction.

1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative, S-shaped microaerophilic bacterium with several flagella on one side. Today, 30-80% of the world's population is infected with this organism and the incidence of infection increases with age. *H. pylori* has been known as the major cause of peptic ulcer, gastric adenocarcinoma and MALT lymphoma(1, 2), currently *H. pylori* positive patients are treated with two antibiotics in addition to a Proton Pump Inhibitor (PPI) and bismuth subsalicylate. These two antimicrobial agents are chosen from the four agents: Metronidazole , Tetracycline, Amoxicillin and Clarithromycin (3).

Despite the use of various regimens for the eradication of *H. pylori* infection, some cases fail to respond to treatment. One of the major causes of infection is resistance to antimicrobial drugs(4).

Studies show that drug resistance to met-

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ronidazole is considerably greater than clarithromycin and resistance to clarithromycin is also more than amoxicillin (1, 5, 6). Multi Drug Resistance (MDR) phenomenon is also observed in some cases, considering that two or all three of the antibiotics can be involved in MDR. (5).

Reduction of nitro group in Metronidazole will produce mutagenic products which are essential for its antimicrobial effects. RdxA protein is an oxygen insensitive NADPH nitroreductase, which reduces many nitroaromatic compounds such as metronidazole. Resistance due to the mutation in rdxA gene could be one principal reason of metronidazole resistance (7-10).

Among phenotypic susceptibility testing methods, disk diffusion test is the easiest and most economical method, but it's not recommended for bacterial species which grow slowly.

Epsilometer agar diffusion gradient test (E-test) is a quantitative variant of disk diffusion method which has shown excellent correlation with agar dilution method for most of antibiotics (11-14). E-test is recommended as the best and simplest method for routine antibiotic susceptibility evaluation for *H.pylori* (12).

There are some discrepancies between agar dilution method and E-test in the detection of metronidazole resistance (6). Another shortcoming is lack of reproducibility in both methods even in the same laboratory, that could be due to mixed infections with both susceptible and resistant isolates, a phenomenon that occurs in 10% of cases in the developed countries (15).

This study evaluates the efficiency of disk diffusion method in comparison with E test method for metronidazole susceptibility testing of *H. pylori* isolates. correlation between deletion of rdxA gene and metronidazole resistance has also been studied.

2. Materials and methods

2.1. Bacterial isolates

A total of 84 clinical isolates of *H. pylori*-from antrum and body biopsies of gastric mucosa were collected between June to December 2014. Exclusion criteria were severe systemic diseases (e.g. renal failure or cirrhosis), history of stomach surgery, consumption of antibiotics and bismuth salts during the past four weeks and antiplatelet drugs during the past one week, previous *H. pylori* eradication regimen, patients under hemodialysis, autoimmune diseases, pregnancy and lactation.

The isolates were from dyspeptic patients which endoscopy was indicated for them. All patients whose biopsies were cultured, had been provided written informed consent under protocols approved by local institutional human studies committees of Shiraz University of Medical Sciences.

The biopsies were transported to the laboratory in a transport media (Granulated yeast extract, Brain hearth broth, Distilled water) and plated onto Columbia agar (Difco) supplemented with 10% horse blood, 7% fetal calf serum, 0.25% yeast extract, 5 µg/l amphotericin B, 5 µg/l trimetoprime and 10 µg/l vancomycin. Culture was performed under microaerophilic conditions in anaerobic jars at 37 °C for 2-10 days and checked for presence of growth every 48 h. Macroscopic identification of *H. pylori* was confirmed by modified Gram staining, catalase, oxidase and rapid urease reactions. The isolates were subcultured on Columbia agar containing 10% horse blood for 48-72 h and stored at -70 °C in preservative media containing skim milk, glycerol, yeast extract and inactivated fetal calf serum.

2.2. Determination of Minimal Inhibitory Concentration (MIC)

The MICs for metronidazole were determined by E-test on Columbia agar supplemented with 10% horse blood, 7% fetal calf serum, 0.25% yeast extract, 5 µg/l amphotericin B, 5 µg/l trimetoprime and 10 µg/l vancomycin. Inoculates were prepared from 48 h incubated agar plates, suspended in transport media to achieve a turbidity equivalent to a McFarland opacity standard of 4 Agar plates (diameter 90 mm, thickness 4 mm) were swabbed all over with 150 µl of the suspension and allowed to dry before E-test strips were applied. Plates with strips containing metronidazole were incubated 72 h under microaerophilic conditions as indicated before. Resistance to metronidazole was defined as MICs≥8 mg/l (16). *H. pylori* CCUG38770, kindly provided by F.M´egraud, was used as the control strain(16).

2.3. Disk diffusion test

Plates were inoculated as described for the E-test and disks containing 5 µg metronidazole (Sigma) were placed on the surface (one disk per plate). Then, the plates were incubated for 72 h at 37 °C under microaerophilic conditions. Inhibition zone diameters were measured in millimeters.

2.4. Primers design

The rdxA gene was detected in the reaction mix using the following primers (17, 18): rdxA1 (5′-AATTTGAGCATGGGGC-GA-3′) and rdxA2 (5′-AAACGCTTGAAAA-CACCCT-3′).

Analysis of primers by BLAST software showed that both primers had 100 % homology with *H. pylori* genome and could identify different strains (Table 1).

2.5. DNA extraction and amplification

DNA was extracted from all 84 *H. pylori*-isolates by boiling and the use of phenol-chloroform methods. The expected fragment was 850 bp if the gene was normal but 650 bp if the gene was mutated. PCR optimization was performed using various amounts of magnesium chloride ($MgCl_2$), dNTPs, primers and different annealing temperatures using several dilutions of template DNA. PCR condition was as follows: reactions were carried out in Eppendorf AG 22331 thermo cycler in 50 µl mixtures containing 5 µl PCR buffer, 2 µl $MgCl_2$, 2 µ dNTP mix, 0.12 µl Taq DNA Polymerase, 34 µl sterile deionized water, 5 µl template DNA and 1 µl of each oligonucleotide primers. Initial denaturation was performed at 94 °C in 5 mins followed by 30 cycles of denaturation at 94 °C in 40 sec, annealing was done in 40 sec at 58 °C, extension at 72°C in 1 min. The final extension step was extended to 10 min at 72 °C.

2.6. Electrophoresis

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

2.7. Statistical analysis

The correlation of the results of disc diffusion and PCR methods were compared by Pearson correlation test. Data were analyzed by SPSS version 18.0. To describe the prevalence of different qualitative variables, descriptive indexes were used. Also mean and standard deviation (SD) were used to present quantitative variables. Significance was defined as a P value of <0.05.

3. Results

From 223 patients referred to the endoscopy unit of "Nemazee hospital" in Shiraz, Iran,

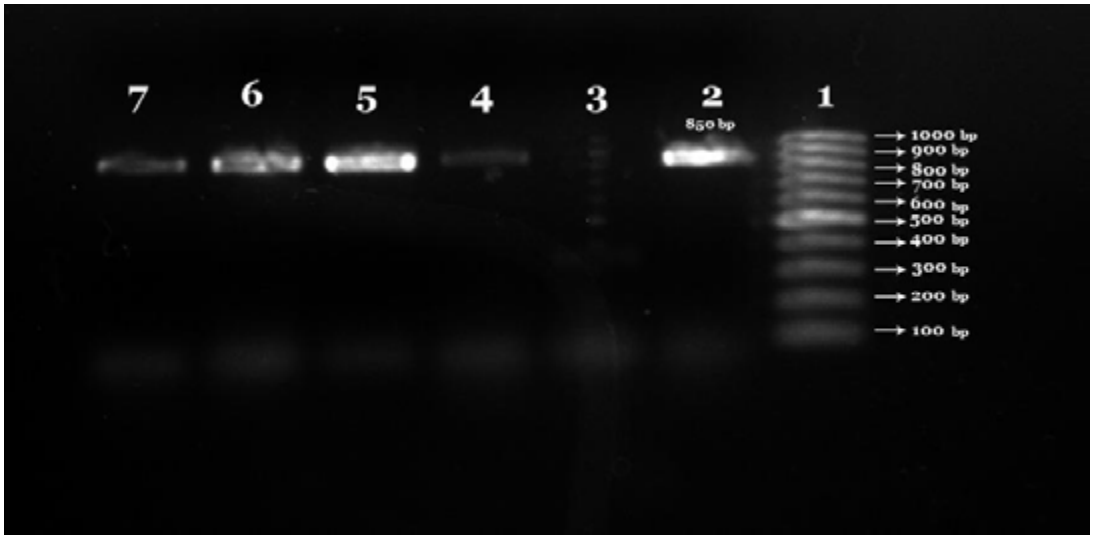

Figure 1. Electrophoresis of rdxA gene products. Number 1:100bp DNA Ladder, Number 2: Positive control, Number 3: Negative control.

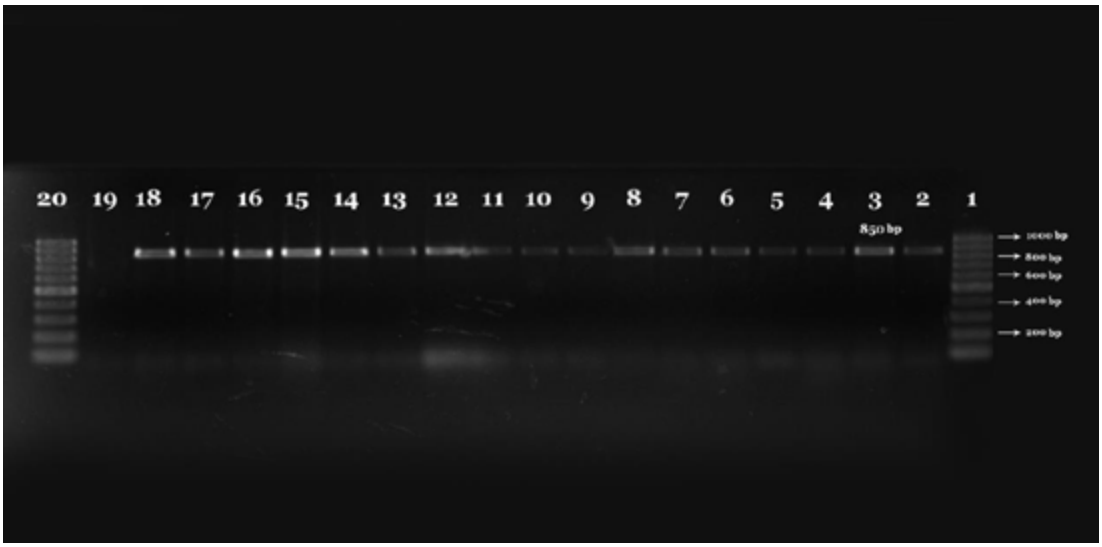

Figure 2. Electrophoresis of rdxA gene products. Number 1 and 20: 100 bp DNA Ladder, Number 2: Positive control, Number 19: Negative control.

55% (115 cases) were female and 45% (108 cases) were male. This population was grouped into three categories: 1) 19-30 years old (25.5%), 2) 30-40 years old (33.9%), 3) 41-65 years old (40.5%). The *H. pylori* isolates obtained from 19 male and 23 female patients (42 *H. pylori* isolates totally). 47.6% and 64.3% of isolates were resistant to metronidazole according to disk diffusion test & E-test respectively. There was a significant relation between gender and metronidazole resistant in the E-test method (P: 0.038) (Table 2). The MIC range in susceptible strains was 0.25-6 µg/ml and in resistant strains was 12 to ≥256 µg/ml.

Sixty two of 65 samples were also analyzed by PCR but none of them showed 850 bp deletion.(Figure 1 and 2) The correlation between deletion of rdxA gene and metronidazole resistance was not significant (P>0.05).

4. Discussion

The results show that 47.6% and 64.3% of the isolates in this study were resistant to metronidazole by the disk diffusion and E-test method respectively. Despite the significant progress that has been made with respect to development of tests to detect antimicrobial resistance, additional work is needed to optimize the performance of the susceptibility testing methods of *H. pylori*, especially for metronidazole. Some studies consider the disk diffusion method as a reliable, cheap and easy method (19) but some other do not recommend this method because of its high error percentage (20, 21). The percentage of resistance in the disk diffusion method has been reported less than the E-test method; 47.6% versus 64.3%. Positive Predictive Value and Negative Predictive Value for disk diffusion was calculated to be 80% and 50% respectively. Also the sensitivity was 59.3% and specificity was 73.3%. In a comprehensive research, McNulty *et al*., have studied factors that may affect different results in other researches(22). Differences in culture medium, size of inoculums, anaerobic pre-incubation and age of cultures are the main reasons of paradoxical results in studies.

The present study determined that there are significant differences in resistance to metronidazole based on gender. It was observed that women are four times more likely than men to have a chance of being resistant (OR=0.25, 95% CI:0.07-0.95).The higher resistance rate to metronidazole in females reported in this and other studies could be due to the treatment of gyneco-

Table 1. Forward and reverse primers.

Expected product size(bp)	Reverse primer	Forward primer	Gene
rdxA	5′-AATTTGAG-CATGGGGCAGA- 3′	5′-GAAACGCTTGAAAA-CACCCCT-3′	851

Table 2. Metronidazole resistance pattern based on gender.

Number (Percent)	Disc Diffusion	E-test
Male	9 (47.3%)	9 (47.4%)
Female	11 (47.8%)	18 (78.3%)
P value	0.976	0.038
Total	42	42

logical infections using this drug which is also used in the treatment of bacterial vaginosis (23).

Rate of resistance to metronidazole in our study is similar to some reports from a number of studies in developing countries which reported 66-100% resistance to metronidazole (24, 25). It appears that the extensive use of 5-nitroimidazole for gynecological and oral infections in the past and parasitic infections in present has contributed to the resulting rate of resistance. Of course the results largely depend on the method used to test the sensitivity(6). It can be seen that even in one region using the same measuring method, pattern of resistance may vary over time, as in a study by Farshad.*et al* conducted in 2010, rate of metronidazole resistance by E-test was reported to be 44% (23). Also, most patients with *H. pylori* infection in this study were 40-65 years old which is comparable with other studies in Asia (26, 27).

None of the resistant or sensitive samples possessed rdxA gene deletion. In a study by Mohammadi.et al in 2005, rdxA gene deletion mutation was seen in only 5% of the strains that metronidazole resistance was detected according to the results of antibiogram (28). In a study by Abdollahi *et al*., only 22.9% of resistant samples showed rdxA deletion mutation(29), In this study also only 2% of resistant samples showed deletion mutation(30). In agreement with the results of other studies, this study indicated the absence of mutations in rdxA in metronidazole resistant clinical samples. According to these results it seems that some other nitroreductase enzymes are involved in metronidazole activation and more complex mechanisms are related with resistance. In a study in 2003 by Marais *et al*., it was shown that even in the absence of rdxA gene activation and function, *H. pylori* strains can be sensitive to metronidazole (9).

In conclusion, using rdxA as a marker gene for the detection of metronidazole resistance at least in southern Iran is not a good method, and more extensive studies should be made on the genetic mechanism of resistance to metronidazole. Although the use of the disk diffusion method for the determination of antimicrobial resistance of *H. pylori* to amoxicillin or clarithromycin, is reliable and inexpensive but this method is not valid for metronidazole. Therefore, considering the increasing resistance rate in many countries, monitoring susceptibility of *H. pylori* to antibiotics appears to be necessary in order to choose effective therapy to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure. Finally, taking into account the present findings along with other reported findings, continued surveillance of the resistance profiles and the resistance mechanisms present in *H. pylori* strains isolated in Iran is essential, if therapeutic plans are to satisfy the country's needs.

Conflict of Interest

None declared.

5. References

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