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Full Length Research Paper

Genetic Variability of *Helicobacter pylori* in Iran: Clarithromycin Resistance and 23S rRNA Mutations

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To determine the 23S *rRNA* point mutations in clarithromycin resistance of *Helicobacter pylori* strains isolated from southwest, Iran. This was a cross-sectional survey, which was done on 263 patients who referred to endoscopy department of Shehrekord university of medical sciences. According to gram stain, urease, catalase, oxidase and polymerase chain reaction (PCR) *H. pylori* identified. Standard National Committee for Clinical Laboratory Standard (NCCLS) method used for assessment of clarithromycin resistance. Specific primers and restriction enzymes *BsaI* and *MboII* by PCR-RFLP were used for analysis of A2143G and A2142G mutations. So for the detection of A2142C, specific primers and PCR method were used. 84 strains of *H. pylori* (31.94%) determined by PCR method. Of 19 (22.62%) clarithromycin resistant strains 13 (68.40%), 3 (15.78%), 2 (10.52%) had A2143G, A2142G, A2142C respectively and one unknown mutation in 23S *rRNA* gene. Because of considerable resistance to clarithromycin, direct diagnosis of this mutation by molecular approach in other parts of the country is necessary.

Key words: Polymerase chain reaction, clarithromycin, resistance, 23S rRNA, polymerase chain reaction - restriction fragment length polymorphism.

INTRODUCTION

Helicobacter pylori, a human-specific colonizer of gastric mucosal epithelium, is associated with a variety of gastrointestinal diseases, including gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphatic tissue lymphoma (Blaser and Atherton, 2004). *H. pylori* infection is difficult to eradicate. According to the Maastricht 2000 Consensus Report, the recommended first-line eradication therapy, especially for patients with peptic ulcer disease, should be based on the combination of a proton pump inhibitor, clarithromycin and amoxicillin or metronidazole (Malfertheiner et al., 2002). In recent years, however, resistance to clarithromycin and metronidazole has become increasingly common. It is current opinion that such resistance is the main cause of the failure of *H. pylori* eradication therapy, although additional factors, such as poor compliance and inappropriate treatment are also implicated (Glupzynski et al., 2001). The failure of a clarithromycin-based regimen can lead to the development of secondary clarithromycin resistance (Gasbarrini et al., 2000). Resistance to clarithromycin is due to the point mutations in the peptidyl transferase-encoding region of 23S *rRNA* which affects the binding of macrolides to the bacterial ribosome (Fluit et al., 2001). Three major point mutations in domain V of the *23S rRNA* gene have been linked to macrolide resistance; A2142G, A2143G and less frequently, A2142C (Alarcon et al., 2000). The purpose of this study was to determine the prevalence and mechanism of clarithromycin resistance in *H. pylori* strains isolated from biopsy specimens, in Chaharmahal and Bakhtiari province, southwest of Iran.

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Abbreviations: RUT, Rapid urease test; **PU,** peptic ulcer; **PCR,** polymerase chain reaction; **RFLP,** restriction fragment length polymorphism.

MATERIALS AND METHODS

Patients

263 consecutive patients with dyspeptic symptoms attending the endoscopy suite of gastroenterology section of Hospital of unoversity of medical sciences, Shahrekord, Iran from July to December 2007 were enrolled. Every patient history sheet was examined in detail and findings were recorded on standard performa including demographic data. Three biopsy specimens for each patient, 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 Shahrekord Azad University. A rapid urease test for the detection of urease activity was performed on biopsy samples.

Bacteria and culture conditions

Biopsy samples were cultured on *Brucella* agar (Merck) supplemented with 7% fresh horse blood, vancomycin (6 mg/L) (Merck), trimethoprim (5 mg/L) (Merck) and amphotricin (2 mg/L) (Merck). Plates were incubated at 37°C in a microaerophilic atmosphere ($5\%O_2$, $15\%CO_2$, $80\%N_2$), for 3 to 5 days for primary culture. Every 2 to 3 days isolates were subcultured in nonselective medium such as brain heart infusion agar containing 7% defibrinated horse blood. Strains were identified according to colony morphology, gram stain and positive reaction with urease, catalase, oxidase. The final test that confirmed *H. pylori* strains was polymerase chain reaction (PCR) for detection *ureC* gene.

Antimicrobial susceptibility testing

The susceptibilities of the *H. pylori* isolates were examined by an agar dilution method. Briefly, isolates were grown for 48 h in brain heart infusion with 10% fetal calf serum, and a suspension of 10^9 CFU/ml was applied to plates containing antibiotic by using a steers replicator. Serial dilutions of the antibiotic ranging from 0.016 to 128 mg/liter were prepared in Muller-Hinton agar supplemented with 10% horse blood. Plates were incubated for 3 to 5 days, and the minimum inhibitory concentration (MIC) was recorded as the lowest concentration of the antibiotic inhibiting visible growth. Resistance breakpoint for metronidazole was defined as the >4 µg/liter (Washington et al., 2006).

DNA extraction and PCR assays

The extraction of *H. pylori* genomic DNA was performed as reported previously (Alarcon et al., 2000). The *ureC* gene was detected by using the primers F-5'AAGCTTTTAGGGGTGTTAGGGGTTT-3' and R-5'AAGCTTATTTCTAACGC-3' with 35 cycles of 94°C for 1 min, 55°C 1 min ,and 72°C for 1 min, which amplifies a 249-bp amplicon. PCR reaction was carried out in eppendorf mastercycler gradient (eppendorf, GmbH, Hamburg, Germany) (Smith et al., 2004). Seven microliter portions of the PCR products were then analyzed by electrophoresis within a 1.5% agarose gel in 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). A2142G and A2143G mutations were determined by a PCR-RFLP method using primers Cla18 (5'-AGTCGGGACCTAAGGCGAG-3') and Cla21 (5'-TTCCCGCTTAGATGCTTTCA-3') (Alarcon et al., 2000; Cavallaro et al., 2006). The amplified fragment was digested with *MboII* (Fermentase) or *BsaI* (Fermentase), which allows discrimination among the wild type, A2142G mutant (*MboII* restriction site), and the A2143G mutant (*BsaI* restriction site) (Figure 1) . The restriction products were analyzed by electrophoresis on a 2% agarose gel. A 3'-mismatch PCR was developed using specific primers Cla3 (5'-AGGTCCACGGGGTCTTG-3') and Cla18 for PCR amplification, approximately 5 ng of genomic DNA of *H. pylori* was added to a 0.2 mM deoxnucleoyide triphosphate, 1M (each primer), and 1.25U of Taq DNA polymerase (Promega) (Alarcon et al., 2000). Amplification was carried out in a DNA eppendorf mastercycler gradient (eppendorf, GmbH, Hamburg, Germany). Thirty five cycles, each for 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C, were performed after 10 min of denaturation at 95°C. A 700-bp fragment was detected with a 1.5% agarose gel only when the 23S *rRNA* gene had the A2142C mutation (Alarcon et al., 2000). Statistical data analysis was performed using Chi-square and Fisher's exact test in the software SPSS version 14.0.

RESULTS

Clinical features

Abdominal pain was present in 76.05% (200/263), dyspepsia 7.61% (20/263), vomiting 6.08% (16/263), heartburn in 6.46% (17/263), and weakness 3.80% (10/263) . The main endoscopic findings were gastritis 36.49%, duodenal ulcer 13.30%, gastric ulcer 9.50%, esophagitis 9.48%, and 5.32% gastric cancer.

Culture, RUT and PCR of biopsy specimens

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

Prevalence of clarithromycin resistance

According to agar dilution method, 19 (22.62%) strains were resistant to clarithromycin. A 1.4kb fragment of 23S *rRNA* gene was amplified in all strains studied here in. Furthermore, *MboII* cut the fragment obtained in 3 strains and *BsaI* cut the other 13 amplified fragments of resistant strains. In the one strain, neither *MboII* nor *BsaI* was able to digest the amplified fragment was obtained by A2142C. Specially primed mismatched PCR, indicating that the strains had the A2142C mutation Figure 2. The results showed no correlation between the clarithromycin resistance and patient gender. There was no correlation between A2142G, A2143G and A2142C mutations. There was significant difference between clarithromycin resistance and A2143G mutation ($p = 0$).

DISCUSSION

Clarithromycin is a key component of most current triple

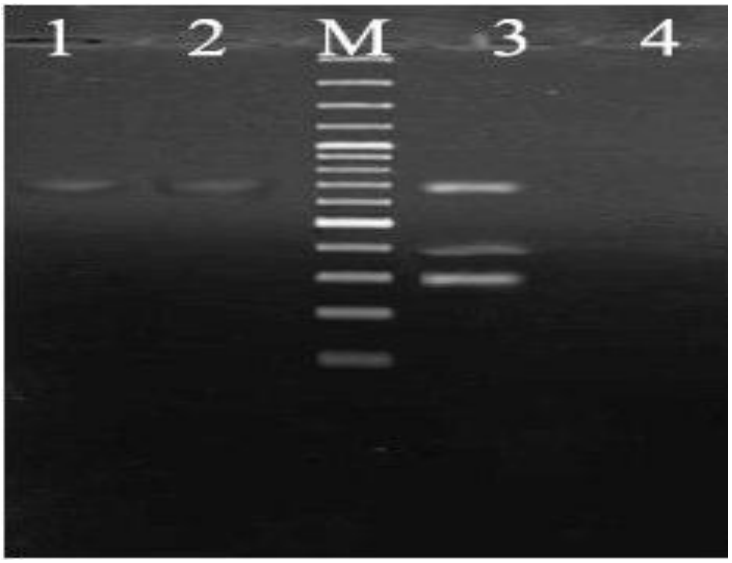

Figure 1. PCR-RFLP pattern obtained after gigestion with MboII and BsaI. Lanes 1 and 2: MboII digetion producing 700bp fragment. Lane M: siz marker 100-3000bp. Lane 3 BsaI digetion producing, 700, 400 and 300bp product. Lane 4: negative control.

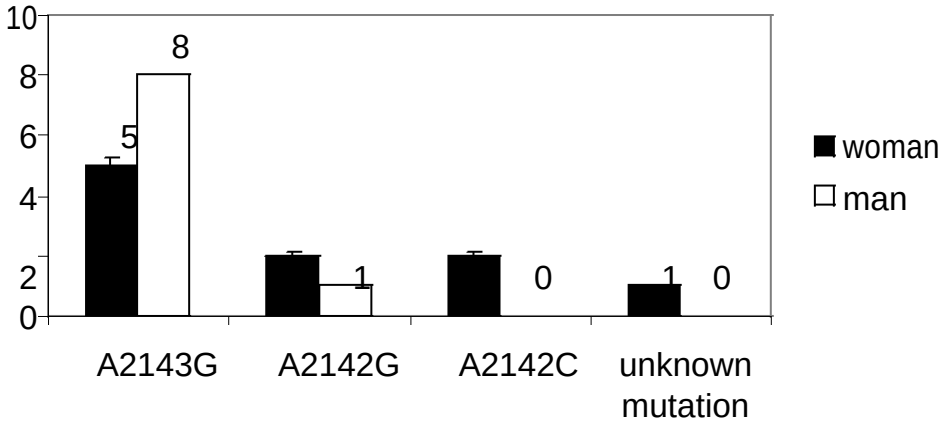

Figure 2. 23S *rRNA* mutations obtained by PCR-RFLP.

therapy regimens for treatment of *H. pylori* infection; however resistance is a major determinant in a failure of eradication regimens (Cavallaro et al., 2006). Rates of resistance to clarithromycin vary widely from region to region. It has been reported that great variations in the resistance rates exist between the northern and the southern parts of Europe. In fact, the rate of resistance to clarithromycin in adults is lower than 5% in northern Europe (Glupczynski et al., 2000). Lower rates of resistance to clarithromycin have been reported from Canada. In fact, it was below 4% in Canada and reached only 10 to 15% in the United States (Fallone, 2000). The prevalence was reported to be 2.14% in Malaysia (Ahmad et al., 2009), 11 to 12% in Japan (Prez Aldana et al., 2002), 4.5% in Hong Kong (Ling et al., 2002), 5 to 6% in Korea (Kim et al., 2001). Prevalence of clarithromycin resistance was 4.16% in 2003 (Fallafi and Maleknejad, 2007) and reached to 23% in 2007 (Tomatari et al., 2010) in Tehran (capital of Iran). In the present study, 22.62% of the isolates were resistant which is equal to resistance rate of Tehran in 2007.

The primary risk factor for resistance to clarithromycin is previous use of macrolides. Excessive uses of macrolides for respiratory tract infections in children increase the risk of resistance to macrolides. Soon after the introduction of macrolides in 1997, resistance to these antibiotics was detected in Estonia in 1998 (Loivukene et al., 2002). There can be cross-resistance

to macrolides. This is particularly important for erythromycin. In fact, when the rate of resistance to clarithromycin was 17% in Tehran, clarithromycin was not being used there, but erythromycin was being used (Mohamadi et al., 2003).

Versalovic et al. (1996) have shown that A2142G and 2143 mutations of 23S *rRNA* gene in *H. pylori* are associated with resistance to clarithromycin. A mutagenesis study performed by Taylor et al. (1997) has confirmed that the A2142G and A2143G mutations are associated with clarithromycin resistance of *H. pylori*. The most frequent mutation was A2143G (69.8) but its prevalence varied from 53 to 95%, followed by A2142G (11.7%) and A2142C (2.6%) (Megraud, 2004). In our study of 19 (22.62%) clarithromycin resistance strains 13 (68.40%), 3 (15.78%), 2 (10.52%) had A2143G, A2142G, A2142C respectively and one unknown mutation in 23S *rRNA* gene. In conclusion resistance to clarithromycin is the main predictor of failure for eradication treatments using this compound, and the detection of resistance is becoming of major importance. PCR-RFLP can be used directly with biopsy specimens, thereby avoiding the requirement for time-consuming culture-based methods. This is particularly important for patients in whom a first eradication attempt has failed.

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