Full Length Research Paper

Molecular assessment of clarithromycin resistant *Helicobacter pylori* strains using rapid and accurate PCR-RFLP method in gastric specimens in Iran

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Currently, a seven-day, triple-drug regimen has been recommended as one of the first-line therapies for *Helicobacter pylori* management in which clarithromycin is a key component. Development of clarithromycin resistance leads to the long term assessment of the efficacy of clarithromycin in the triple-drug regimen. The aim of this study was to rapidly and directly assess clarithromycin resistance point mutations on gastric biopsy specimens by using PCR-RFLP method. Biopsy samples were obtained over a 6-months period of 2009, from 200 dyspeptic patients referred to Shahrekord University of Medical Sciences, Iran. Initially, rapid urease test was performed and then DNA was isolated from each tissue and used for molecular analysis such as PCR (for *H. pylori* diagnostic) and PCR-RFLP (for Cla resistance determination). RUT and PCR results showed that 164 (82%) of the patients were *H. pylori*-positive. Resistance was evaluated in 164 samples by using enzymes *BsaI* and *MboII*. Thirty nine (39) (23/78%) clarithromycin-resistant strains were detected which were identified as 15 (9.15%) A2143G, 15 (9.15%) A2142G and 9 (5.49%) mix strains. The results showed that PCR-RFLP method had a high accuracy to detect A2142G and A2143G mutations associated with resistance to clarithromycin in the minimum possible time.

Key words: *Helicobacter pylori*, clarithromycin resistance, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

INTRODUCTION

*Helicobacter pylori* infects about 50% of the world's population and is thus a major source of gastritis, gastric ulcer, duodenal ulcer and an important risk factor for gastric cancer (Alarcon, 2000, 2003; Fontana, 2003; Lee et al., 2005; Kargar, 2010). It is widely known that all patients with gastric or duodenal ulcer and *H. pylori* infection should be treated with antimicrobial agents, since eradication of the bacteria cures peptic ulcer disease and efficiently prevents relapses (Lee, 2005; Liu, 2008; Lottspeich, 2007; Occhialini, 1997).

The most advocated therapy, triple therapy, often includes clarithromycin (Fontana, 2003; Occhialini, 1997). However, clarithromycin resistance in *H. pylori* has been shown to occur at different rates (1 to 10%) in different countries and is an important cause of the failure of these regimens (Occhialini et al., 1997). The mechanisms of clarithromycin resistance have been elucidated and consist of a mutation in the functional domains of the 23S rRNA in *H. pylori*, which is located in domain V. In particular, the main 23S rRNA mutations are an adenine-to-guanine transition at positions 2142 and 2143. These single point mutations also generate specific restriction sites, namely *BsaI* and *MboII*, which can be used for the rapid screening of clarithromycin resistance (Alarcon, 2003; Fontana, 2003; Lee, 2005). Successful detection of
these mutations in cultured strains or gastric biopsy specimens has been described by the use of fluorescent in situ hybridization, PCR-restriction fragment length polymorphism, reverse hybridization line probe assay, PCR and EIA of DNA, and several real-time PCR methods (Lottspeich et al., 2007). The aim of this study was to rapidly and directly assess clarithromycin resistance point mutations on gastric biopsy specimens by using PCR-RFLP method.

**RESULTS**

Detection of *H. pylori* directly in gastric biopsy samples

RUT results showed that 164 (82%) of the patients were *H. pylori*-positive. DNA samples were derived from gastric biopsy specimens of confirmed *H. pylori*-positive patients which were positive by the diagnostic PCR assays for the 16S rRNA and 23S rRNA targets. Both PCR assay confirmed the presence of *H. pylori* in all the 164 biopsy samples (100%) and generated the expected PCR product of 109 and 1400 bp in 16S rRNA and 23S rRNA PCR (Figure 1).

**DISCUSSION**

All 164 cases found to be *H. pylori* positive by the RUT, were also PCR positive. It is important to emphasize that the PCR assay confirmed all results from phenotypic test (Lottspeich et al., 2007).

Following the recognition of the important pathogenic role of *H. pylori* infection in the development of gastro-duodenal diseases, there has been a continuous search for improved eradication therapy. Clarithromycin emerged as one of the antibiotics of choice because of its low MIC, which is relatively unaffected by lowering the pH, as well as its high concentration in gastric mucosa. In binding experiments, the tightest interaction for a macrolide-ribosome complex observed to date was found for the binding of clarithromycin to *H. pylori* ribosomes. However, clarithromycin used as a single antibiotic cannot eradicate more than 70% of strains, and resistant strains have been isolated from patients who were not cured. When a second antibiotic was added, the success rate increased to about 90% but resistant strains were still isolated from case failures (Occhialini et al., 1997).

*H. pylori* high resistance to clarithromycin correlated...
completely with A2142G and A2143G transition mutations in the 23S rRNA gene, resulting in a decrease in the affinity of clarithromycin to bind to ribosomes. The high incidence of clarithromycin resistance in adults may reflect frequent prescriptions of macrolides for treatment of respiratory tract infections other than H. pylori. Some investigators have recently reported the predominance of an A2143G mutation in primary resistance isolates in Europe. This predominance was not shown in Korean and Brazilian study, and the A2142G mutation was predominant. Since the H. pylori genome is known to contain a high degree of genetic variability, Iranian H. pylori isolates may be similar to Western isolates of H. pylori and different from Korean and Brazilian isolate (Lee, 2005; Ribeiro, 2003).

Treatment failure should prompt endoscopy, culture and susceptibility test. Retreatment should exclude antibiotics with acquired resistance. Many studies have highlighted the difficulties in retreatment, and it can be stated that the best available first-line treatment regimen is still the best rescue treatment. The H. pylori eradication rate was significantly improved when antibiotic therapy was performed on the basis of the results of antimicrobial susceptibility test (17 to 18% improvement in different report) (Lee et al., 2005).

Resistance to clarithromycin is the main predictor of failure for eradication treatments using this compound, and the detection of resistance is mainly important. Detection of resistance in H. pylori is generally performed by MIC determination. This method requires growth of bacteria and takes at least 10 days; thus, the applicability of the data in the clinical setting is sometimes controversial. PCR-RFLP method provides a result within few hours once the strain has been isolated, and no special technology apart from PCR is needed. The results could be more practical if the method were applied directly to biopsy specimens, which would provide a faster result (Alarcon et al., 2000).

Unlike indirect assays such as ELISA and 13C-UBT, PCR assays are direct assays to detect H. pylori infection. Thus, these assays should result in higher specificity than the indirect assays. H. pylori colonizes on the apical surface of stomach’s epithelial cells and sheds into gastric juice with the regular renewal of gastric mucosa. Several PCR assays based on H. pylori DNA from gastric juice/biopsy sample were reported for the detection of H. pylori infection. The possible H. pylori-specific target genes included ureA, ureB, ureC, ureD, 16S rRNA and 23S rRNA. Point mutations of 23S rRNA were closely related to formation of clarithromycin resistance (Liu et al. 2008). Since the PCR product of 23S rRNA described in this study could be used as a biomarker to detect H. pylori infection and clarithromycin resistance simultaneously, this assay has advantages over other PCR-based methodologies. However, the specificity and sensitivity of the 23S rRNA PCR assay was not evaluated extensively. No single test is accepted as the standard for diagnosis of H. pylori infection.

We have assessed a simple, rapid and cost-effective procedure which can detect H. pylori in gastric biopsy specimens with good sensitivity and evaluated the clarithromycin resistance of the microorganism. The second aspect of the development of this procedure was

**Figure 1.** 109 bp PCR product of 16S rRNA PCR for confirming H. pylori in gastric biopsy specimens. (M): 100 bp marker (100 to 1000 bp, Fermentas GMBH, Germany), (1): Positive control (obtained from Biotechnology Research Center of Islamic Azad University, Shahrekord Branch), (2 to 4): H. pylori positive samples and (5): Negative control (NTC).

**Figure 2.** RFLP result. (1): A2142G positive control; (2) A2142G negative control; (3) A2142G positive strain; (4): A2143G positive control; (5): A2143G negative control; (6): A2143G positive strain. Final lane is 100 bp marker (100 to 3000 bp, Fermentas GMBH, Germany). All positive controls are obtained from Biotechnology Research Center of Islamic Azad University, Shahrekord Branch and negative controls are susceptible strains.
the combination of knowledge, concerning all restriction sites associated with 23S rRNA mutations (those associated with high resistance phenotypes) and therefore clarithromycin resistance. The result has been rapid endonuclease restriction analysis of the amplicons which is easy to perform and does not present any difficulty in interpretation.

In conclusion, our PCR detection of H. pylori in gastric biopsy specimens is reliable and easy to perform and can provide additional information, specifically related to the macrolide susceptibility of the microorganism. Therefore, the extensive use of this method not only isolate H. pylori infecting the patient, but also direct the first eradication therapy or evaluation of the outcome of previous eradication regimens of the patient (with a consequent reduction in cost).

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REFERENCES


