African Journal of Biotechnology Vol. 10(39), pp. 7675-7678, 27 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2607
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

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

Mohammad Kargar[1]*, Sadegh Ghorbani-Dalini[2], Abbas Doosti[3] and Maryam Baghernejad[1]

[1]Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran.
[2]Jahrom Branch, Young Researcher's Club, Islamic Azad University, Jahrom, Iran.
[3]Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.

Accepted 10 June, 2011

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 *Bsa*I and *Mbo*II. 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 *Bsa*I and *Mbo*II, which can be used for the rapid screening of clarithromycin resistance (Alarcon, 2003; Fontana, 2003; Lee, 2005). Successful detection of

*Corresponding author. E-mail: mkargar@jia.ac.ir.

Abbreviations: RUT, Rapid urease test; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; ***H. pylori***: *Helicobacter pylori*; **RUT,** rapid urease test.

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.

MATERIALS AND METHODS

A total of 200 patients with a median age of 52.5 years (range, 17 to 88 years) presenting with upper gastrointestinal symptoms during June 2009 to November 2009, were enrolled in the study. All patients read and signed an 'informed consent' form at the beginning of endoscopy and declared their satisfaction with the application of their anonymous data for research purpose.

A rapid urease test (RUT) for the detection of urease activity of *H. pylori* was performed with a Gastro urease kit (Bahar-afshan, Iran) on biopsy samples. DNA was isolated from each tissue with a DNA extraction kit (DNP^{TM}, CinnaGen, Iran) according to the manufacturer's instruction and immediately used for molecular analysis such as PCR (for *H. pylori* diagnostic) and PCR-RFLP (for clarithromycin resistance determination).

H. pylori detection in gastric biopsy samples by PCR

H. pylori was detected with the PCR amplification method for a portion of the *16S rRNA* gene. The reaction was performed directly on eluates obtained from gastric sample purification. One microliters of purified DNA was added to each PCR amplification reaction. The reaction was performed with a couple of primers whose nucleotide sequence was derived from a known sequence of the *16S rRNA* gene: HP-1 forward (5′-CTGGAGAGACTAAG CCCTCC-3′) and Hp-2 reverse (5′-ATTACTGACGCT GATTGTGC-3′) (Alarcon et al., 2000). Amplification reaction mixtures (24 µl) contained 1 µl of extracted DNA, 200 mM (each) deoxynucleoside triphosphates (dNTPs) (dNTP Mix, CinnaGen, Iran), 0.2 mM (each) primer (CinnaGen, Iran), 1.5 mM $MgCl_2$ and 1 U of *Taq* polymerase (CinnaGen, Iran) in PCR buffer (CinnaGen, Iran). Amplification was carried out in an Eppendorf master cycler gradient (eppendorf, Germany). Thirty (30) cycles, each consisting of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C, were performed after 5 min of denaturing at 95°C. Cycles were followed by a final elongation at 72°C for 5 min. The Hp16S fragment was visualized after electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

PCR-RFLP analysis

A 1,400-bp fragment of the *23S rRNA* gene was amplified with primers Cla-18 (5′-AGTCGGGACCTAAGGCGAG-3′) and Cla-21 (5′-TTCCCGCTTAGATGCTTTCAG-3′). PCR amplification of DNA was performed in a final volume of 24 µl PCR mixture, containing 2 µl of extracted DNA, 200 mM of each dNTPs, 0.2 mM of each primers, 1.5 mM $MgCl_2$ and 1 U of *Taq* polymerase in PCR buffer. Amplification was carried out in an Eppendorf master cycler gradient over 30 cycles, each for 1 min at 95°C, 1 min at 62°C and 1 min at 72°C. These cycles were performed after a denaturation of 5 min at 95°C and a final elongation step at 72°C for 5 min.

The amplicon was digested with *BsaI* (Fermentas GMBH, Germany) for 1 h at 37°C and *MboII* (Fermentas GMBH, Germany) for 1 h at 37°C to detect the restriction site occurring when the mutation was A to G at 2143 or 2142, respectively. The restriction products were analyzed by electrophoresis on a 2% agarose gel.

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 samples 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).

PCR-RFLP

Thirty-nine (39) resistant strains were detected by PCR-RFLP and were distributed as follows: 15 pure A2143G genotype, 15 pure A2142G genotype, 6 wild type/ A2143G genotypes, one wild type/A2142G genotype and two A2143G/A2142G genotypes. *BsaI* cuts the PCR product of the wild-type sequence into two fragments of 1,000 and 400 bp and that of the A2143G sequence into three fragments of 700, 400 and 300 bp. *MboII* cuts the PCR product into two fragments of 700 bp only when A2142G was present in the sequence (Figure 2).

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

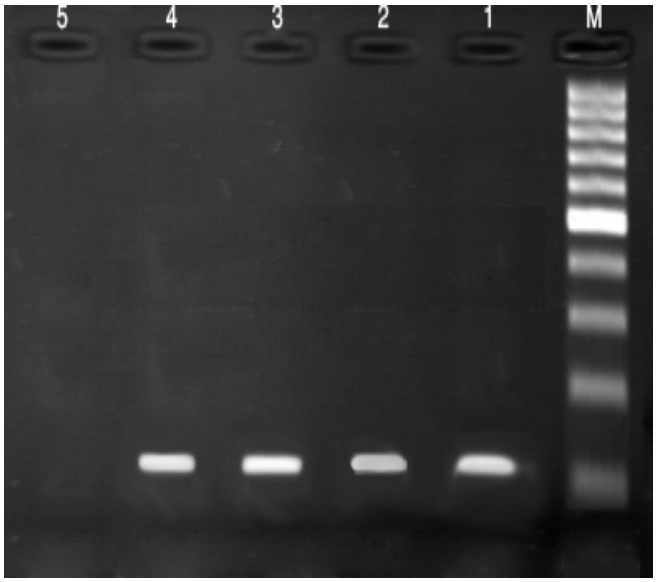

Figure 1. 109 bp PCR product of *16S rRNA* PCR for confirming *H. pylori* in gastric biopsy specimens. (M): 100 bp marker (100 to 1000 bp, Fermentas GMBH, Germany), (1): Positive control (obtained from Biotechnology Research Center of Islamic Azad University, Shahrekord Branch), (2 to 4): *H. pylori*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

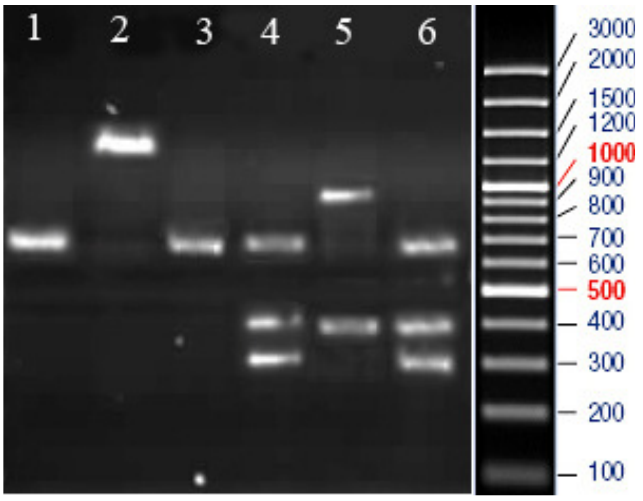

Figure 2. RFLP result. (1): A2142G positive control; (2) A2142G negative control; (3) A2142G positive strain; (4): A2143G positive control; (5): A2143G negative control; (6): A2143G positive strain. Final lane is 100 bp marker (100 to 3000 bp, Fermentas GMBH, Germany). All positive controls are obtained from Biotechnology Research Center of Islamic Azad University, Shahrekord Branch and negative controls are susceptible strains.

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 ^{13}C-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

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).

ACKNOWLEDGEMENTS

The authors are grateful to the Islamic Azad University, Jahrom and Shahrekord branches, for their executive support of this project.

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