

# SURVEY IN IRAN OF CLARITHROMYCIN RESISTANCE IN *HELICOBACTER PYLORI* ISOLATES BY PCR-RFLP

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**Abstract.** The aims of this study were to assess primary resistance of *H. pylori* strains isolated from adult patients of Ilam, Iran to antibacterial agents (amoxicillin, clarithromycin, metronidazole and tetracycline) and detection of clarithromycin, azithromycin, clarithromycin, metronidazole and tetracycline resistance by disc diffusion. Fifty biopsies were taken from gastric mucosa of the antrum and body regions of adult patients by gastroscopy, and were cultured on *Helicobacter pylori* selective medium. The susceptibility of *H. pylori* strains showed that 44, 6, 6, 4 and 16% were resistance to metronidazole, amoxicillin, tetracycline, azithromycin, and clarithromycin, respectively. Polymerase chain reaction- restriction fragment length polymorphism analysis showed that all clarithromycin resistance isolates had A2143G mutation and PCR amplicons from these strains upon digestion by *Bsa*I restriction enzyme resulted in 319 and 106 base pair fragments. Because most of physicians in Ilam do not use amoxicillin in triple therapy of *H. pylori* infection, isolates showed low rate of resistance to amoxicillin.

**Keywords:** clarithromycin resistance, *H.pylori*, PCR-RFLP, Iran

## INTRODUCTION

*Helicobacter pylori* is a gram-negative spiral gastroduodenal pathogen that colonizes the human stomach and is found in more than half of the world's population. Although most of *H. pylori* infections are asymptomatic, but it can also cause severe gastro-duodenal diseases including chronic gastritis, peptic ulcer disease, and increase the chance of development of gastric cancer (Agudo *et al*, 2010; De

Francesco *et al*, 2011; Tanih *et al*, 2011).

It is suggested that *H. pylori* infection in patients with symptoms must be eradicated (Agudo *et al*, 2010). Standard therapy combines a proton pump inhibitor (PPI) or ranitidine bismuth citrate and two antibiotics, chosen from among amoxicillin, clarithromycin, and metronidazole (Agudo *et al*, 2010). Although these triple-drug regimens are usually effective in eradication of *H. pylori* infection, increase of resistance, especially to clarithromycin and metronidazole among clinical *H. pylori* strains, is able to sharply decline the treatment rate of *H. pylori* infection and is one of the major factors responsible for the failure of eradication (Cerqueira *et al*,

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2011; Karczewska *et al*, 2011; Liu *et al*, 2011; Tanih *et al*, 2011).

Prevalence of *H. pylori* resistance varies around the world, and it has been related to rate of antibiotic consumption in the general population (De Francesco *et al*, 2010a). However, clarithromycin is the key drug of triple therapies and several studies have demonstrated that primary resistance to clarithromycin has become one of the most important reasons for treatment failure (Agudo *et al*, 2010). The rate of *H. pylori* resistance to clarithromycin varies among different countries, such as 16% in Japan, 1.7% to 23.4% in Europe and 10.6% to 25% in North America (Agudo *et al*, 2010). Clarithromycin is a macrolide antibiotic, acts by binding to the peptidyltransferase region of 23S rRNA and inhibits protein synthesis. The resistance to clarithromycin in *H. pylori* has been shown to be due to distinct point mutations within the peptidyltransferase region encoding in domain V of *H. pylori* 23S rRNA gene (Agudo *et al*, 2010; Ben-Mansour *et al*, 2010; De-Francesco *et al*, 2011). The most common mutation is an A-to-G transition at position 2143 (A2143G), but other mutations, A2142G, A2144G, A2142C, and T2182C, have been demonstrated. Other mechanisms of resistance, such as methylase production, the action of macrolide-inactivating enzymes, and active efflux pumps, have been reported in several bacteria (Ben-Mansour *et al*, 2010; De Francesco *et al*, 2010b). One method for detecting 23S rRNA gene mutations is based on the generation of restriction sites for restriction enzymes (Versalovic *et al*, 1996; Taylor *et al*, 1997; Sevin *et al*, 1998).

The aim of this study was to assess primary resistance of *H. pylori* strains isolated from adult patients of Ilam, Iran to antibacterial agents (amoxicillin, clarithromycin, metronidazole and

tetracycline) and detection of clarithromycin resistance gene mutation by PCR-RFLP.

## MATERIALS AND METHODS

### Patients

Fifty patients who presented with dyspepsia were selected for this study. In an outpatient clinic in Ilam city, during January 2009 to March 2010, the patients who underwent gastroscopy were biopsied to obtain specimens from gastric mucosa of the antrum and body regions for culturing of *H. pylori* and testing of drug susceptibility. These patients included 22 men and 28 women. The biopsies for culture were placed into sterile saline solution for transport to Department Microbiology, Ilam University of Medical Sciences. Informed consent was obtained from each patient before being included in this study, which was approved by the Ethics Committee of Ilam University of Medical Sciences, Iran.

### Culture of biopsies and antibiotic susceptibility

Biopsy samples were processed in less than 3 hours from the time that the biopsy specimens were obtained. Biopsy samples were cultured on Brucella agar plates supplemented with 10 % sheep blood and Skirrow supplement (Oxoid, Cambridge, England) and incubated under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>) for 7 days at 37°C. Isolates were identified as *H. pylori* on the basis of the macroscopic appearance of colonies (gray, small, and translucent colonies on blood agar), positive biochemical reactions (oxidase, catalase, and urease), and additionally Gram staining of the colonies to verify gram-negative curved bacteria.

The susceptibilities of *H. pylori* strains to amoxicillin, metronidazole, clarithro-

Table 1  
Resistance to antimicrobial agents by  
*H. pylori* isolates.

Antibiotics	Resistance %
Metronidazole	88% (n = 44)
Amoxicillin	12% (n = 6)
Tetracycline	12% (n = 6)
Azithromycin	8% (n = 4)
Clarithromycin	32% (n = 16)

mycin, tetracycline and azithromycin were assessed quantitatively by disc diffusion (Fermentas, Mamheim, Germany). Disc diffusion was performed on Muller Hinton agar after inoculation of culture adjusted to 0.5 McFarland turbidity by insertion of each strip on the medium. The results were analyzed after 24 hours.

#### DNA extraction

The total bacterial genomic DNA of the 50 isolates was extracted using a commercial DNA extraction kit (Fermentas) according to the manufacturer's instructions, and stored at -20°C for further analysis.

#### PCR-RFLP for detection of point mutations in *H. pylori* 23S rRNA gene

Presence of point mutations in the domain V of *H. pylori* 23S rRNA gene (A2143G and A2142G) were performed by PCR-RFLP method. PCR amplification using the primers Hp23-1 (5' CCACAGC-GATGTGGTCTCAG-3') and Hp23-2 (5'-CTCCATAAGAGCCAAAGCCC-3') (Pina *et al*, 1998) flanking a region of 425 bp within the bacterial 23S rRNA peptidyltransferase (Hp23S fragment) was performed with denaturation at 94°C for 5 minutes, followed by 30 cycles according to the following program: 94°C for 1 minute, 67°C for 1 minute, 72°C for 1 minute, and a final heating of 10 minutes at 72°C.

For detection of point mutations, we used *BbsI* and *BsaI* enzymes (Fermentas) for detection of A2142G and A2143G mutation, respectively. Finally, digested PCR and non- digested PCR products were visualized under UV-illuminator after electrophoresis in 2% agarose gel stained with ethidium bromide.

## RESULTS

#### Antibiotic susceptibility by disc diffusion

The susceptibility of 50 *H. pylori* strains showed that 44, 6, 6, 4 and 16% were resistance to metronidazole, amoxicillin, tetracycline, azithromycin, and clarithromycin, respectively (Table 1). Among them, 22 isolates were resistant to metronidazole, which were isolated from 16 women and 6 men. Only 2 and 3 cases were resistance to azithromycin and tetracycline respectively, all isolated from women. Eight isolates resistant to clarithromycin were isolated from 5 women and 3 men. Ten cases were sensitive to all 5 antibiotics, and 40 isolates were resistant to one or more antibiotics tested in this study. Among the resistant isolates, 6 were resistant to both clarithromycin and metronidazole, and 2 were resistance to three antibiotics including amoxicillin, clarithromycin and metronidazole.

#### Determination of point mutations in *H. pylori* 23S rRNA gene by PCR-RFLP

After PCR amplification using primers specific for the 23S rRNA gene (Hp23S fragment), and electrophoresis of PCR products by agarose gel stained with ethidium bromide, a 425 bp band was obtained. PCR products were separately digested by *BbsI* and *BsaI* enzyme. All clarithromycin resistant isolates had point mutation A2143G, but A2142G and dual (A2143G and A2142G) mutations were not observed (Fig 1).

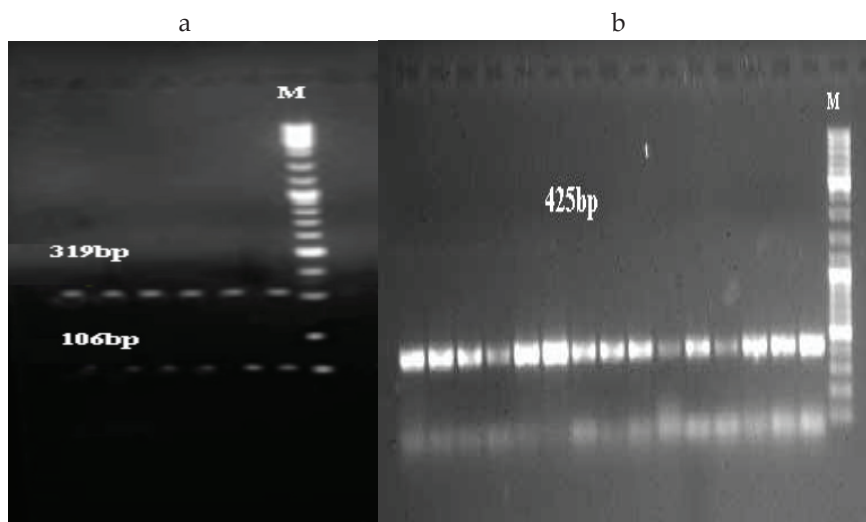


Fig 1-PCR-RFLP analysis of *H. pylori* 23S rRNA for detection of A2143G mutation. Digestion by *Bsa*I enzyme results in the generation of 319 bp and 106 bp bands (a) from 425 bp band (b).

## DISCUSSION

Antibiotic resistance in *H. pylori* strains especially to metronidazole and clarithromycin, two most important antibiotics in eradication of infection of this bacteria, is a worldwide challenge and the level of primary clarithromycin resistance is increasing worldwide. Resistance to clarithromycin, the most common antibiotic against *H. pylori* infections in triple therapies, has an important role in causing failure of eradication. For this reason, detection of susceptibility of *H. pylori* to antibiotics around the world can help to gain a better understanding of the effect of resistance on therapy outcome.

In this study a high rate of resistance to metronidazole was obtained. Our results confirmed previous reports on the high prevalence of metronidazole-resistant strains in Iran and other developing countries. Different prevalence of resistance to metronidazole, 23, 40.5, 54 and 73.4%, were reported in previous

studies in Iran (Falsafi *et al*, 2004; Fallahi and Maleknejad, 2007; Abadi *et al*, 2010; Shokrzadeh *et al*, 2011). According to reports from around the world, various ranges of resistance especially to metronidazole and clarithromycin have been reported, for example in one study in Kuwait, 70% of isolates were resistance to metronidazole and also high resistance rate was reported in South Korea (Albert *et al*, 2006; Kim *et al*, 2006).

In our study, 22 isolates of *H. pylori* were resistant to metronidazole, and these strains were isolated from 16 women and 6 men, indicating a significant association between rate of metronidazole resistant in *H. pylori* strains and sex ( $p < 0.05$ ). High resistance to metronidazole can be explained to be due to using metronidazole in eradication of genital infections (parasitic and bacterial infections) in women.

Resistance to amoxicillin was obtained in 6% of isolates, in agreement with the low rate of resistance of 2.4, 6.8,

and 8.33% reported in previous studies in Iran (Fallahi and Maleknejad, 2007; Abadi *et al*, 2010; Shokrzadeh *et al*, 2011). In Europe and USA, resistance rates are less than 1%. In contrast, high resistance rates have been reported in South Korea (18.5%) and in Indonesia (19.4%) (Mégraud, 2004; Kim *et al*, 2004; Kumala and Rani, 2006). However, in one report from Kuwait, amoxicillin-resistant *H. pylori* was not found (Kim *et al*, 2006).

In this study, clarithromycin was the second most resistant antibiotic, 8 (16%) of the isolates. Different resistance rates to clarithromycin were reported in Iran, *viz* 2.4, 4.16, 14.3, 16 and 30% (Sivoshi *et al*, 2006; Fallahi and Maleknejad, 2007; Rafeey *et al*, 2007; Abadi *et al*, 2010; Shokrzadeh *et al*, 2011). Reports from developed countries showed that approximately 10% of *H. pylori* strains were clarithromycin-resistant, but in developing countries, this resistance rate is higher (Alarcon *et al*, 1999; Bindayna, 2001; Yilmaz and Demiray, 2007). Because of current usage of clarithromycin for treatment of *H. pylori* in Iran, this resistance may be related to cross resistance from other macrolide antibiotics, such as erythromycin and clarithromycin (Falsafi *et al*, 2004).

The resistance to clarithromycin in *H. pylori* occurs due to distinct point mutations in domain V of the *H. pylori* 23S rRNA gene (Agudo *et al*, 2010). A review by Mégraud (2004) of several studies worldwide has shown that 81.5% of the mutations in clarithromycin-resistant isolates are the A2142G or A2143C mutation.

Our study is second report of the detection of the point mutations in the 23S rRNA gene of *H. pylori* in Iran. In this study, all clarithromycin resistant isolates had only the point mutation A2143G,

and no point mutations were detected in clarithromycin-susceptible strains of *H. pylori*. In one study in Iran, 93.7% of *H. pylori* A2143G mutation was reported (Abadi *et al*, 2011). In a study from Uruguay all clarithromycin resistant isolates have A2143G mutation (Torres-Debat *et al*, 2009). In our study resistance to tetracycline was 6%, and resistance to this antibiotic from 0 to 9% were reported in Iran (Fallahi and Maleknejad, 2007; Abadi *et al*, 2010; Shokrzadeh *et al*, 2011). It has been reported that primary resistance to tetracycline is rare in *H. pylori* isolates (Mégraud, 2004).

In summary, we observed a clarithromycin resistant prevalence of 16% in *H. pylori* strains of Iranian patients with dyspepsia and PCR-RFLP results showed that all clarithromycin resistant isolates continued A2143G mutation in 23S RNA gene.

#### ACKNOWLEDGEMENTS

This study was supported in part by Ilam University of Medical Sciences, Iran.

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