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Clarithromycin Resistance and *23S rRNA* Mutations in *Helicobacter pylori*

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1. Introduction

Helicobacter pylori infects about 50% of the world's population and is a major cause of chronic gastritis, is strongly associated with the development of gastric and duodenal ulcers and has been linked with gastric adenocarcinoma and B-cell mucosa-associated lymphoid tissue lymphoma [1, 2].

H. pylori Infection results in a sequence of events, ultimately resulting in the development of some gastrointestinal disorders. The sequence was first suggested by Correa et al. in 1975 and has since been supported by many other studies. Colonization of the gastric mucosa by *H. pylori* first lead to the induction of an inflammatory response, predominantly by Th1 (T helper cells type 1). The initial acute gastritis is followed by active chronic gastritis, which lasts for life if the infection is not treated. Nevertheless, *H. pylori*-positive subjects are mostly unaware of this inflammation due to the lack of clinical symptoms. The Th1 response results in epithelial cell damage rather than in the removal of *H. pylori* because *H. pylori* is not an intracellular pathogen. The ongoing presence of *H. pylori* thus causes a lifelong proinflammatory response coupled to cellular damage and initiates the histological cascade. The continuous production of reactive oxygen species that results from the ongoing inflammation can result in DNA damage, thus inducing the multiple mutations thought to be required for initiation of the cancer cascade depicted in Figure 1 (3, 4).

Among the new methods of magnifying endoscopy, a prototype of endocytoscopy developed by Olympus was used for ex vivo visualization of *Helicobacter pylori* on experimentally infected gastric biopsies. Moving bacteria were observed at 1100× magnification, giving hope for a possible direct detection during endoscopy. Kim et al. also used magnifying endoscopy on 103 patients to classify the gastric surface according to four patterns: flat, irregular, papillary or nonstructured, which were then compared to the updated Sydney System for histologic gastritis. Histologic gastritis was found in 91% of the biopsy sections with a nonflat type, and among them, 96% were confirmed to harbor *H. pylori* infection. In another study, the magnified endoscopic findings in the gastric body

were classified into four patterns and then correlated with histology results. Type 1 pattern corresponded to normal gastric mucosa, types 2 and 3 to *H. pylori* -infected mucosa and type 4 to atrophy. The sensitivity and specificity for these endoscopic findings were 92.7% and 100% for type 1, and 100% and 92.7% for types 2 and 3 together, respectively (5).

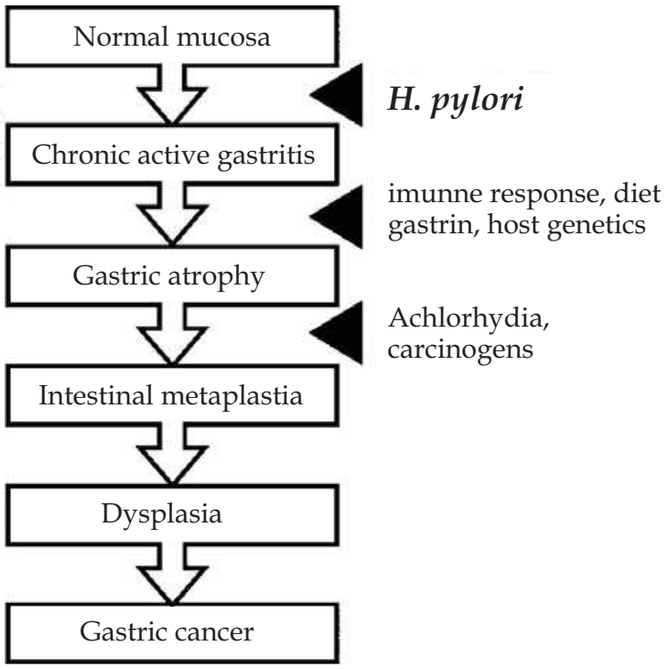

Fig. 1. Model representing the role of *H. pylori* and other factors in gastric carcinogenesis, based on the cascade proposed by Correa et al (3, 4)

2. *H. pylori* detection

Various diagnostic assays for the detection of an *H. pylori* infection are available. Histological detection and culturing of the pathogen are gold standard, which require invasive gastroduodenoscopy to obtain gastric biopsy specimens. In the last decade, noninvasive approaches, such as serological detections, the [^{13}C] and [^{14}C]urea breath test (UBT), and detection *H. pylori* antigen or DNA in feces, helped and improved the evaluation of *H. pylori* infection status in patients. Because of low sensitivities of most serological assays for younger than 12 years of age patients, they are not suitable for pediatric. The UBT is a well-established noninvasive diagnostic assay and gives excellent performance for both adults and children, but its specificity decreases for infants and young children and need. In addition, the performance of UBT with infants and young children requires trained staff for air sampling with a face mask, and the test also requires expensive instruments, such as an isotope ratio mass spectrometer or an infrared isotope ratio spectrometer. Enzyme immunoassays (EIAs) for the identification of *H. pylori* antigens in fecal specimens circumvent these difficulties. EIAs based on monoclonal antibodies have shown consistent excellent results, with very high sensitivities and specificities for both adults and children. A major disadvantage of all the noninvasive tests described above is their inability to provide information on the susceptibility or resistance of *H. pylori* to antibiotics [2].

Methods		Sensitivity and Specificity	Typical application
Invasive methods	Histology	>95%	Gold standard in routin hospital diagnostics
	Biopsy culture	>95%	Alternative gold standard
	RUT	>90%	Cost-effective and rapid test
Noninvasive methods	UBT	>95%	Alternative gold standard
	Fecal antigen	>90%	Not widely used yet
	Serology	80-90%	Mainly used for epidemiological studies

Table 1. Non-PCR base diagnosis of *H. pylori* infection (3)

3. Macrolide resistance

H. pylori infection can be cured by antibiotics, however the ideal anti-*H. pylori* treatment has yet to be found. Many factors have been implicated in treatment failure, including ineffective penetration of antibiotics into the gastric mucosa, antibiotic inactivation by low gastric pH, lack of compliance, and emergence of acquired antibiotic resistance by *H. pylori*. Despite the success of the current anti-*Helicobacter* therapies, it is suggested that eradication rates among patients with gastritis are lower than among patients with peptic ulcer disease, with the causes of this phenomenon still being the subject of speculation [6].

The macrolide class of antimicrobial agents is over 30 years old and is still at the forefront of antimicrobial therapy as well as drug discovery and development. Clarithromycin is a recently approved 14-membered macrolide with increased stability in acid and improved pharmacokinetics, including the appearance of a microbiologically active metabolite in humans. Clarithromycin possesses broad-spectrum antimicrobial activity, inhibiting a range of gram-positive and gram-negative organisms, some anaerobes, and atypical pathogens, in many cases with greater in vitro activity than erythromycin [7].

Clarithromycin is a semi-synthetic macrolide antibiotic. Chemically, it is 6-*0*-methylerythromycin. The molecular formula is $C_{38}H_{69}NO_{13}$, and the molecular weight is 747.96 (Figure 2). Clarithromycin is a white to off-white crystalline powder. It is soluble in acetone, slightly soluble in methanol, ethanol, and acetonitrile, and practically insoluble in water.

Currently, a seven-day, triple-drug regimen has been recommended as one of the first-line therapies for *H. pylori* management. This treatment includes omeprazole (a proton-pump inhibitor), clarithromycin, and amoxicillin or metronidazole [2, 8]. However, this therapy is being investigated because of increased eradication failures due to the prevalence of clarithromycin resistant *H. pylori* infections. Many studies have shown that between 0–50% of *H. pylori* isolates were clarithromycin resistant, which leads to a need for long term

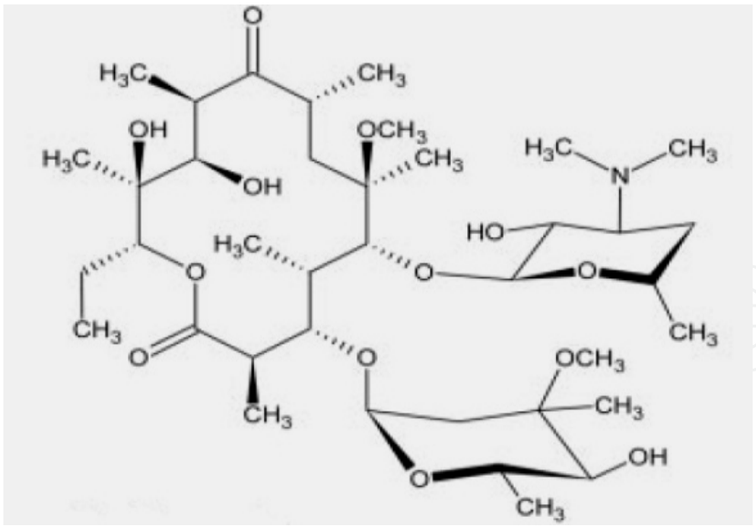

Fig. 2. The structural formula of clarithromycin

assessment of the efficacy of clarithromycin in the triple-drug regimen. It is well known that the abuse of macrolide antibiotics including clarithromycin might lead to clarithromycin resistant forms of *H. pylori* [8]. Clarithromycin is a bacteriostatic antibiotic, which belongs to a group of macrolides bound to peptidyltransferase loop of domain V and VI of the 23S rRNA molecule (Figure 3 and 4). This binding interferes with protein elongation, and thus effectively blocks bacterial protein synthesis.

The antibacterial activity of clarithromycin is similar to that of other macrolides, but clarithromycin is better absorbed in the gastric mucus layer, more acid-stable, and therefore more effective against *H. pylori*. Resistance to clarithromycin is thought to develop when substitutions in one nucleic acid at or near this binding site on the ribosome prevent the drug from binding, thereby making it ineffective [9]. Mutations A2144G, A2143G, A2142G and A2143C are the most often observed and reported by investigators, other mutations such as A2142C, A2115G, G2141A, A2142T and T2717C have been described but appear to be rare in peptidyltransferase [10, 11].

4. Detection of clarithromycin resistance

Many diagnostic assays have been developed for *Helicobacter pylori*: culture, histology, rapid urease test, urea breath test, serology, stool antigen test, and molecular-based tests [2, 5, 10, 11, 15]. Culture has the great advantage of permitting subsequent determination of the antimicrobial susceptibility of the strain isolated, in particular to macrolides. However, disadvantages of culture include special conditions for specimen transportation, the use of complicated media with special conditions for maintenance, the need for special incubation conditions, and the length of time necessary to obtain a result [10]. In routine practice the detection of clarithromycin resistance is mainly based on phenotypic methods performed after culture: agar diffusion for the E-test or the agar dilution method, which is considered the reference; however, these methods are time-consuming [16].

The association between point mutations in the *23S rRNA* gene and macrolide resistance in *H. pylori* potentially provides a new approach for diagnosing macrolide resistant *H. pylori* strains [9]. Numerous molecular-based methods are now available to assess clarithromycin

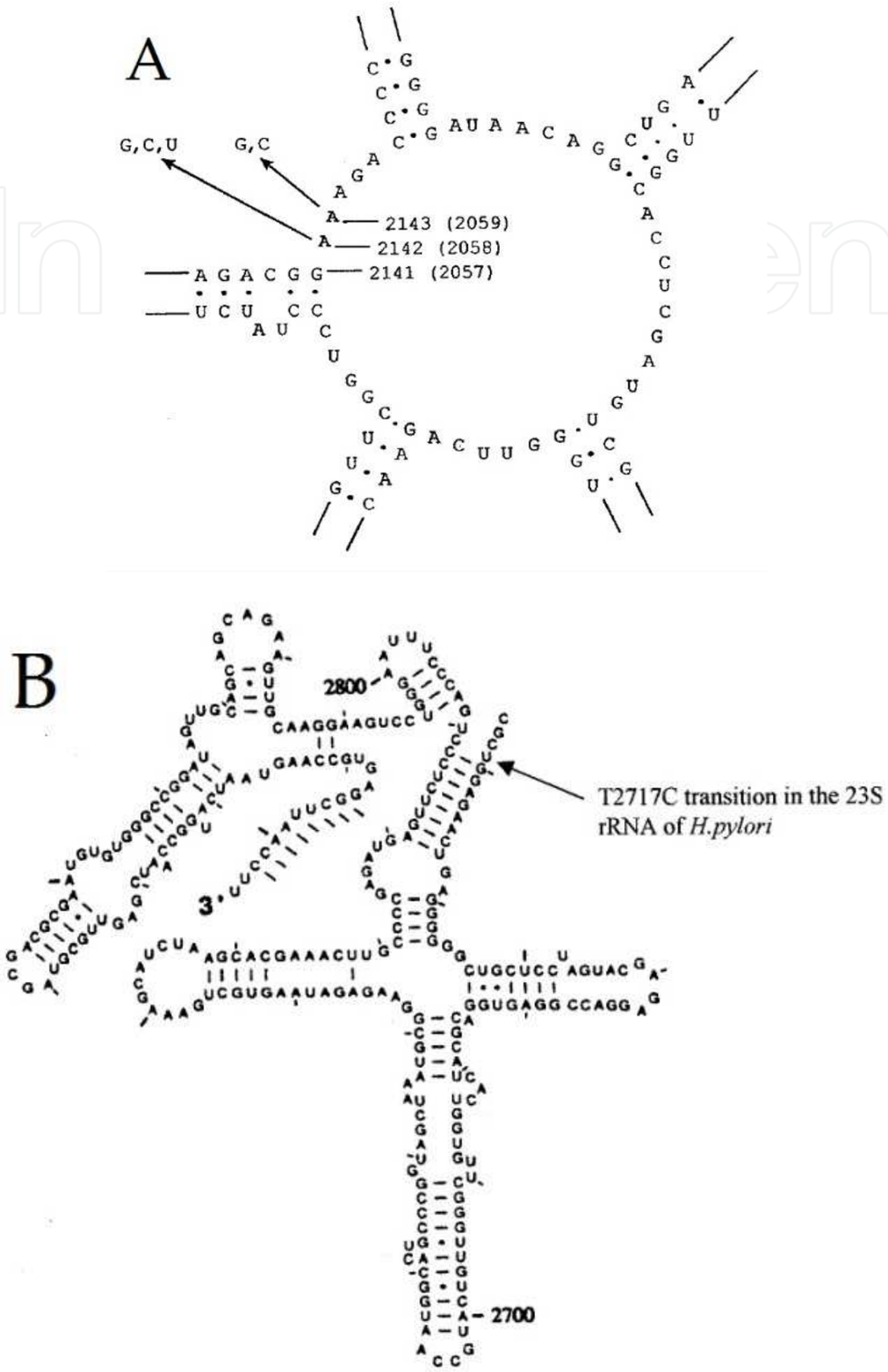

Fig. 3. Model of domain V and VI of 23S rRNA from *E. coli*, (12 , 13)

in *H. pylori*, such as PCR-RFLP, PCR-OLA, PCR-DEIA, PCR- LiPA, PCR-PHFA, 3M-PCR, real-time PCR, FISH, FRET, DNA sequencing by conventional and real-time (pyrosequencing) techniques [2, 9-11]. Most assays are polymerase chain reaction (PCR)-based using different methods to study the amplicons. The PCR-based molecular techniques are quicker than microbiological susceptibility testing, and more importantly, they can be performed directly on gastric biopsies and gastric juice [9].

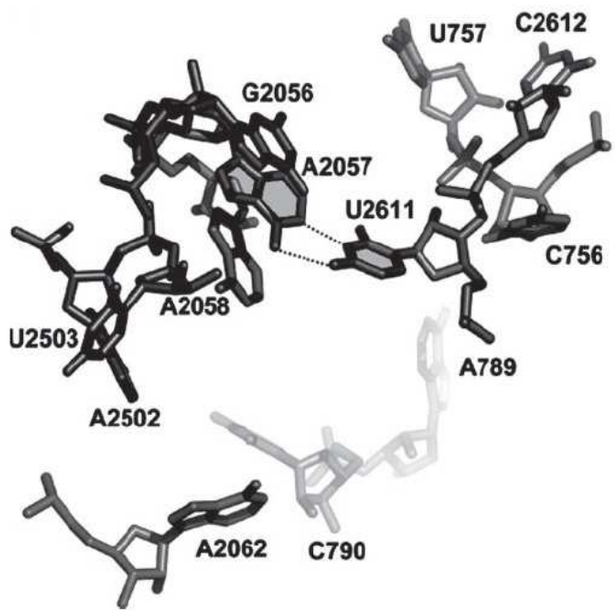

Fig. 4. A view into the macrolide-binding pocket in *E. coli* highlighting the specific geometry of the 2611–2057 base pair. H bonds are shown as dotted lines. The macrolide-binding pocket is located at the upper part of the ribosomal peptide exit tunnel, at a distance from the peptidyl transferase center that allows the accommodation of polypeptides of 5–6 residues. It is formed mainly by 23S rRNA domain V nucleotides, among which A2058 and A2059 play a prominent role in binding, selectivity, and resistance. (14)

A PCR-based approach to diagnostic testing has the advantage over existing non-culture-based tests in that it is simple to perform and can provide additional genotypic information about the infecting strain, including markers associated with antibiotic susceptibilities. In addition, although the initial cost of equipment is high, the cost of reagents and consumables for each test is extremely low in comparison with corresponding costs for other methods such as the urea breath test and stool antigen test [17]. Great advantage of PCR is that it does not require viable bacteria. The transport conditions are thus not as critical as they are for culture, and shipment costs are cheaper. The cost of the reagents necessary for our technique is reasonable: $6.20 for *H. pylori* detection only and $8.60 for detection and clarithromycin susceptibility testing. By microbiological techniques, detection is cheaper, $1.70, but the price of detection with clarithromycin susceptibility testing by E-test, $6.90, is comparable to that of PCR [10]. Nested PCR generally increases sensitivity but also has a high risk of contamination. Real-time PCR has several advantages over conventional PCR, such as short working time, high specificity, and low risk of contamination. Thus far, some studies used real-time PCR targeting either *ureC* or *16S rRNA* and *23S rRNA* for the

quantitative detection of *H. pylori* in gastric biopsies [11]. FISH allowed simultaneous detection of *H pylori* and the point mutations. Also, FISH could be directly applied to formalin fixed tissue sections without extensive preparation of nucleic acid. Another advantage of this technique compared with PCR based systems became obvious when mixed strains and tissue sections harboring more than one *H. pylori* strain were examined. A mixed culture cannot be unequivocally differentiated from a strain carrying two different rRNA operons by restriction enzyme analysis or filter hybridization; this can be done easily by whole cell hybridization technology [18]. PCR-RFLP has some disadvantage than other PCR base techniques including: i: when multiple strains are present in one sample, PCR-RFLP technique can not able to identify genotypes that has low concentration, ii: PCR-RFLP technique can not able to detect two mutations A2144G and A2143C, iii: Mixed populations containing a resistant strain associated with a susceptible strain are difficult to detect by PCR-RFLP. The resistant strain is often well detected because the mutation conferring resistance produces a restriction site that results in a new band on the electrophoresis profile. However, the uncut fragment corresponding to the susceptible clone is usually not taken into consideration. The uncut fragment is indeed more often attributed to an incomplete restriction than to a mixed population. Even for pure resistant strains, a band corresponding to the uncut fragment of DNA is sometimes still present on the electrophoresis profile due to partial restriction. Thus, a mixed population containing a susceptible strain associated with a resistant strain is detected as a single resistant strain [19].

A TaqMan probe is a short oligonucleotide (DNA) that contains a 5' fluorescent dye and 3' quenching dye. To generate a light signal (i.e., remove the effects of the quenching dye on the fluorescent dye), two events must occur. First, the probe must bind to a complementary strand of DNA. Second, *Taq* polymerase, the same enzyme used for the PCR, must cleave the 5' end of the TaqMan probe (5' nuclease activity), separating the fluorescent dye from the quenching dye (Figure 5) (20).

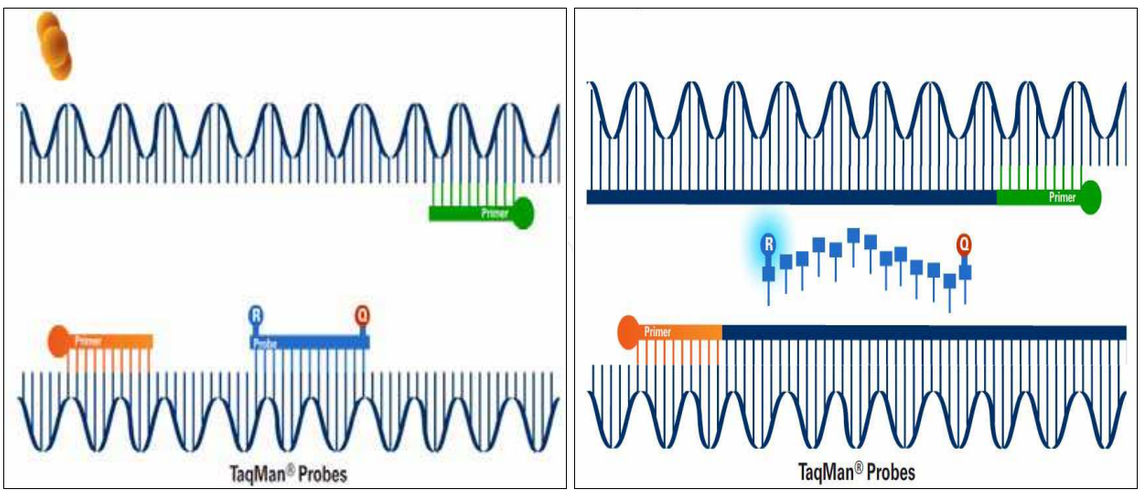

Fig. 5. Schematic model for TaqMan probe technology

Culture has the great advantage of permitting subsequent determination of the antimicrobial susceptibility of the strain isolated, in particular to macrolides. However, disadvantages of culture include special conditions for specimen transportation, the use of complicated media with special conditions for maintenance, the need for special incubation conditions, and the

length of time necessary to obtain a result [10]. Low bacterial density and a patchy distribution of the pathogen may have been the reasons for the negative culture and histology results. This may also be true for the patient determined to be negative by the rapid urease test, histology, and culture but positive by both PCR assays in the biopsy and the stool specimen, thus resulting in a reduction of the PCR specificity to 98% [11]. Culture is only semiquantitative and is time consuming. Histology is also semiquantitative, but its accurateness is relatively weak because of great interobserver variation. Urea breath test has been shown to be uncorrelated to culture-determined bacterial density [10].

5. Epidemiology of clarithromycin resistance

Resistance to clarithromycin is the main predictor of failure of eradication treatments including this compound. Because of an increased use of these macrolides, not only for *H. pylori* eradication, but also for the treatment of respiratory tract infections, the prevalence rate of resistant strains is increasing, and the detection of resistance is becoming of major importance. To render this detection process more effective, results must be available rapidly.

Several studies have shown that the eradication of *H. pylori* plays an important role in the treatment of some gastrointestinal diseases and the results of all currently used tests for bacterial detection can be affected by the treatment [21]. Therefore, a growing attention has been assigned to *H. pylori* antibiotic susceptibility monitoring, due to the increasing prevalence of antibiotic resistance worldwide. Rates of clarithromycin resistance in *H. pylori* strains have been reported in France 21%, Spain 28.3%, Portugal 44.8%, Poland 23.5%, Mexico 21.6%, China 5%, Korea 16.7%, Italy 26.7% and Germany 47% [5, 15, 22-24]. A similar geographical distribution has been observed in Iran, with clarithromycin resistance values ranging from 21% [25] to 35.98% .This relevant discrepancy could be explained not only by the different geographic distribution of resistances, but also by the use of different methodologies to assess clarithromycin resistant status. For example, a study recently performed in the same geographic area (Shahrekord, Iran) has reported clarithromycin resistance rate 22.62% [26] distinctly lower compared with our present experience (35.98%). Nevertheless, it is possible that the different methods used (antibiogram vs. real-time PCR) could have played a role in these discordant data as bacterial culture is hampered by limitations, such as a low sensitivity, even in expert-hands [22].

Although *H. pylori* has two copy of *23S rRNA* gene in own genome, but many authors suggested that more than one strain can present in one patient [11, 27-29]. Also many authors displayed multiple genotypes in one sample because: 1) A troubling aspect of resistance to some antibiotics by *H. pylori* is a phenomenon that has been given the name heteroresistance. This phenomenon has been previously observed for resistance to metronidazole [30]; 2) *H. pylori* is transformable bacterium and the transformation frequency for clarithromycin resistance of *H. pylori* was found to be approximately 2×10^{-6} transformants per viable cell [31]; 3) More importantly, Taylor and et al. suggests that acquisition of antibiotic resistance could result from the horizontal transfer of clarithromycin resistance determinants from resistant cells to susceptible cells of the same strain, probably increasing the population of resistant strains. In addition, such genetic transfer could occur between different strains, since mixed infections with different *H. pylori* strains does occur in some individuals [31].

Country	Year	Technique	Sample No.	Resistance rete	Ref
Australia	2004	Real-time PCR	92	24%	8
France	1994-1999	E-test	150	21%	32
France	1998	Hybridization in Liquid Phase	41	56%	33
France	1999	PCR-DNA Enzyme Immunoassay	61	37.7%	21
France	2000	E-test	61	18%	34
France	2003	Real-time PCR	200	67%	11
France	2003	Real-time PCR	196	18.5%	7
France	2004-2008	Real-time PCR	126	20%	35
Germany	2001	FISH	109	31.2%	36
Germany	1999-2002	E-test	1233	20%	37
Netherland	2001	RAPD-PCR	976	5.2%	8
Italy	2003	PCR-RFLP	283	1.6%	12
Italy	2003	PCR-RFLP & E-test	230	14%	38
Ireland	2001	LiPA	50	26%	39
Spain	2008	E-test	118	35.6%	40
Brazil	2001	Agar Diliution	202	9.85%	41
Brazil	2003	Agar Dilution	155	16%	3
Mexico	1995-1997	E-test	195	24%	42
Peru	1995	Egg Yolk Agar	18	50%	43
Argentina	2000	PCR-RFLP	96	23.9%	44
USA	1996	PCR-OLA	72	55%	45
China	2001	Primer mismatch PCR	96	5%	16
China	200-2009	Agar Dilution	293	8.6%-20.7%	46
Japan	1998	Seminested PCR	85	9%	47
Japan	1999	PCR-RFLP	79	6.3%	22
Japan	2000	PCR-PHFA	412	22%	48
Japan	2001	Real-time PCR	186	21.2%	49
Japan	2001	PCR-RFLP	51	29%	50
Malaysia	2005-2007	E-test	187	2.1%	51
Indonesia	2006	Disk diffusion	126	27.8%	52
Korea	2004	PCR-RFLP	114	20.2%	53
Iran	2007	PCR-RFLP	263	22.6%	54
Iran	2009	PCR-RFLP	200	23.78%	55
Iran	2009	Real-time PCR	200	35.98%	55
Iran	2008-2009	E-test	121	5%	56
Tunisa	2005-2007	Real-time PCR	273	14.6%	57
Cuba	2005	E-test	46	10%	58

Table 2. Prevalence of clarithromycin resistance in the world. Comparison between date, technique and region

Currently, many research aims to evaluation of clarithromycin resistance in *H. pylori* were performed which showed variety of resistance rate around the world, due to 3 main reasons including differences in i: methods, ii: region and iii: date (Table 2).
Many investigators were assessed clarithromycin resistance rate in Europe in 1994 to 2009. In these studies, clarithromycin resistance was more prevalent in France. Also, Fontana et al by PCR-RFLP method showed that clarithromycin resistance was more divers between north (1.6%) and south (14%) of Italy in 2003.
In continent of America, clarithromycin resistance was varies between 9.8% in Brazil to 55% in USA. Less variety were observed in eastern Asia, by 20% to 27% resistance rate. In Iran (Middle East) with variety of customs, Kargar et al showed resistant rate of 23% and 36% in same geographic region in 2009 due to different methods including PCR-RFLP and real-time PCR assays respectively but, PCR-RFLP showed 22% and 23%resistance rate in 2007 and 2009 respectively. Also more variation was observed in 2 near region with different customs and economic level, with 36% in low economic level and 5% in high economic level.

6. Epidemiology of clarithromycin resistance mutations

Many studies aim to assessment of clarithromycin resistance rate were preformed around the world. This studies showed A2144G > A2142G > A2143G > A2142C > A2143C order for clarithromycin resistance point mutations (Table 3).

Researcher	Country	technique	Mutations rate	Ref
Pina	**France**	Hybridization in Liquid Phase	A2144G(56%) A2143G(35%) A2143C(9%)	32
Marais	France	PCR-DNA Enzyme Immunoassay	A2144G(48%) A2143G(24%) A2143C(3%)	21
Russman	Germany	FISH	A2144G(59%) A2143G(35%) A2143C(6%)	36
Maeda	Japan	Seminested PCR	A2144G(87.5%) A2143G(12.5%)	47
Matsuok	Japan	PCR-RFLP	A2144G(80%) A2143G(20%)	22
Matsumura	Japan	Real-time PCR	A2144G(55%) A2143G(5%)	49
Kargar	Iran	PCR-RFLP	A2143G(68.3%) A2142G(15.8%)	54
Kargar	Iran	Real-time PCR	A2144G(5.7%) A2143G(37.1%) A2142G(28.6%) A2143C(21.4%)	55

Table 3. Prevalence of clarithromycin resistance point mutations

7. Problem statement

Following the recognition of the important pathogenic role of *H. pylori* infection in the development of gastroduodenal diseases, there has been a continuous search for improved eradication therapy (Occhilini et al., 1997). *H. pylori* culture, as well as antimicrobial susceptibility studies is difficult to perform as well as labor intensive. Moreover, although the culture method allows antimicrobial susceptibility testing for several antibiotics, only the susceptibilities of macrolides and, in particular, of clarithromycin are really useful since the last is a major predictor of treatment failure. Therefore, detection of clarithromycin-resistant *H. pylori* will facilitate the choice of an appropriate eradication regimen

7.1 Aplication area

The characterization of resistance mechanisms in *H. pylori* and their easy detection will facilitate the choice of appropriate treatment regimens and ultimately the control of infection. 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.

7.2 Material and methods

Study design

In order to assess the clarithromycin resistance rate, 23S rRNA point mutations responsible for clarithromycin resistance and effect of technique, region and date, 2 separate analyses were performed.

7.3 Analysis 1

Patients

263 consecutive patients with dyspeptic symptoms attending the endoscopy center of the gastroenterology department of the Hajar Hospital, Shahrekord, Iran, were enrolled in this study from July to December 2008. Patient-reported symptoms and endoscopic findings of pathologist were recorded at the time of the consultation by the pathologist help, and these data were obtained retrospectively for analysis. The number of participants who were ineligible or declined participation in the study were not recorded.

For the purpose of analysis, three global variables were created: 1) patient-reported included age, gender and symptoms, 2) clinical signs, and 3) clarithromycin resistance data. Patient-reported symptoms included pain, anorexia, heaviness after meal, early satiety, nausea, vomiting and flatulence. Clinical signs were included gastric ulcer, gastric cancer, non-ulcer disease, gastic erosion, nodularity, gastritis and duodenit. All patients read and signed an 'informed consent' form at the beginning of endoscopy declared their satisfaction for application of their anonymous data for research purpose.

Three biopsy specimens were taken from antrum and corpus of each patient, using a disinfected endoscope. Biopsy samples were placed in 0.1 ml of sterile saline solution and sent to the Biotechnology research center of Shahrekord Azad University. A rapid test for the detection of urease activity was performed by Gastro Urease kit (Bahar-afshan, Iran) according to manufacturer's instructions. DNA was isolated from each tissue with a DNA extraction kit (DNP™, CinnaGen, Iran) according to the manufacturer's instruction and immediately used for molecular analysis.

Bacteria and culture conditions

Biopsy samples were cultured on *Brucella* agar (Merck) supplemented with 7% fresh horse blood, vancomycin (6mg/L, Merck), trimethoprim (5mg/L, Merck) and amphotericin (2mg/L, Merck). For primary culture, plates were incubated at 37°C in a microaerophilic atmosphere (5% O_2, 15% CO_2, 80%N_2) for 3 to 5 days. Strains were identified according to colony morphology, Gram staining and positive reactions with urease, catalase, and oxidase. The *ureC* gene (*glmM*) which encodes urease was used as a DNA target to confirm *H. pylori* strain.

Antimicrobial susceptibility testing

The susceptibilities of the *H. pylori* isolates to clarithromycin were examined by an agar dilution method according to *CLSI* (Clinical and Laboratory Standard Institute) protocol (32). Resistance breakpoint for clarithromycin was defined as the >4 µg/liter.

Conventional PCR assays

A PCR assay targeted at the *ureC* (*glmM*) gene of *H. pylori* was performed with specific primer (Table 4) in an eppendorf mastercycler gradient (eppendorf, Germany). Briefly, the 23 µl PCR mixture, containing 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), was held for 5 min at a denaturation temperature of 95°C, followed by 30 cycles of 1 min each at a denaturation temperature of 95°C, an annealing temperature of 58°C, and an elongation temperature of 72°C and by 5 min at 72°C. The amplified 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 and Cla-21 [44] (Table 3). PCR amplification of DNA was performed in a final volume of 24 µl PCR mixture, containing 2 µl of extracted DNA, 200 mM dNTPs, 0.2 mM (each) primer, 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerase in PCR buffer. Amplification was carried out in an eppendorf mastercycler 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* 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 transition at 2143 or 2142, respectively (Figure 5). The restriction products were analyzed by electrophoresis on a 2% agarose gel.

Fig. 5. Schematic diagram for detection of nucleotide alterations of *23S rRNA* by PCR-RFLP assays

7.4 Analysis 2

Sample collection

200 biopsy samples were obtained over a 6-month period (June 2009 to November 2009) from 200 dyspeptic patients referred for endoscopy at Hajar hospital in Iran. All patients read and signed an 'informed consent' form at the beginning of endoscopy declared they are satisfied with application of their anonymous data for research purpose. Every patient history sheet was examined in detail and clinical findings including demographic data were recorded. The mean age of the patients was 52.5 years (range, 17 to 88 years), and 48.1% of the patients were men. Rapid urease test was performed with a Gastro urease kit (bahar-afshan, Iran). DNA was isolated from each tissue with a DNA extraction kit (DNP™, CinnaGen, Iran) according to the manufacturer's instruction and immediately used for molecular analysis. Conventional PCR for detection of *ureC* gene and PCR-RFLP analysis for detection of point mutations were performed as in analysis 1.

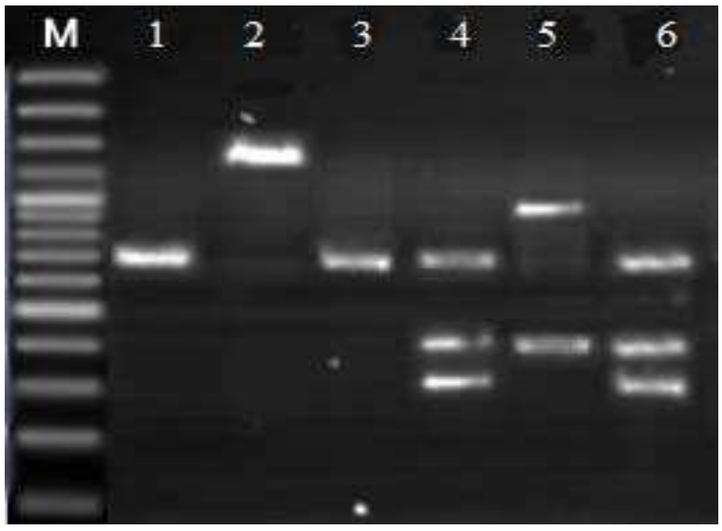

Fig. 6. PCR-RFLP patterns obtained after digestion with *Bsa*I or *Mbo*II. *BsaI* cuts the PCR product of the wild-type sequence into two fragments of 1,000 and 400bp and that of the A2143G sequence into three fragments of 700, 400, and 300bp. *MboII* cuts the PCR product into two fragments of 700bp only when A2142G is present in the sequence: 1)A2142G positive control, 2)A2142G negative control, 3)A2142G positive strain, 4)A2143G positive control, 5)A2143G negative control, 6)A2143G positive strain

Real-time (TaqMan) PCR assay

The real-time PCR was performed by using primer pair HP23S-1 and HP23S-2 and modified probes Pwt, P44G, P43G and P43C that previously reported by Pina et al [33] and newly designed probe P42G according to *23S rRNA* gene sequence (GenBank accession no. U27270) for identification of wild type, A2144G, A2143G, A2143C and A2142G genotypes respectively (Table 3).

The real-time (TaqMan) PCR mixture was prepared until reaching a final solution of 25 µl containing 2 µl of extracted DNA, 200 mM dNTPs, 0.2 mM (each) primer HP23S-1 and HP23S-2, 0.1 mM probe Pwt, 0.2mM probe P44G, 0.1 mM probe P43G, 0.1 mM probe P43C and 0.75 mM probe P42G (Bioneer,Tokyo, Korea) (Table I), 1.5 mM $MgCl_2$, and 1 U of *Taq*

polymerase in PCR buffer 1X. Real-time PCR analysis was performed with an Rotor-Gene 6000 (Corbett Research, Australia). The conditions of PCR amplification were 95°C for 5 min and 45 cycles of 95°C for 30 s and 58°C for 40 s. All samples were repeated twice and positive and negative controls were enclosed in each assay. Data were analyzed with rotor-gene 6000 software *ver1.7* (Corbett research). In order to test the specificity of the primers purified DNA of non-*H. pylori* strains was also used as a template for PCR.

Quantification real-time PCR

In order to made standard serial dilution, standard density of bacteria was prepared. After DNA extraction, one 10-fold serial dilution of *H. pylori* DNA was made, with bacterial concentrations ranging from 5×10^2 to 5×10^7 bacteria per 1 µl. Bacterial quantification was performed by TaqMan probe technology of real-time PCR as described above.

Statistical analysis

Statistical analyses were conducted with *chi-square* using the SPSS for Windows (version 17; SPSS, Inc., Chicago, Illinois, USA). *P*-values less than 0.05 were taken to indicate statistical significance.

Target	Sequence (5′→3′)	Primer/Probe
ureC	AAGCTTTTAGGGGTGTTAGGGGTTT	HP-1
	AAGCTTATTTCTAACGC	HP-2
23S rRNA	AGTCGGGACCTAAGGCGAG	Cla-18
	TTCCCGCTTAGATGCTTTCAG	Cla-21
23S rRNA	CCACAGCGATGTGGTCTCAG	HP23S-1
	CTCCATAAGAGCCAAAGCCC	HP23S-2
Wild type	Cy5-GGGGTCTTTCCGTCT-BHQ2	Pwt
A2144G	TAMRA-GGTCCTTCCGTCTTG-Dabcyl	P44G
A2143G	TET-GGTCTCTCCGTCTTG-Dabcyl	P43G
A2143C	HEX-GGTCTGTCCGTCTTG-Dabcyl	P43C
A2142G	FAM-GGTCTTCCCGTCTTG-Dabcyl	P42G

Table 4. Oligonucleotides sequence used in this study

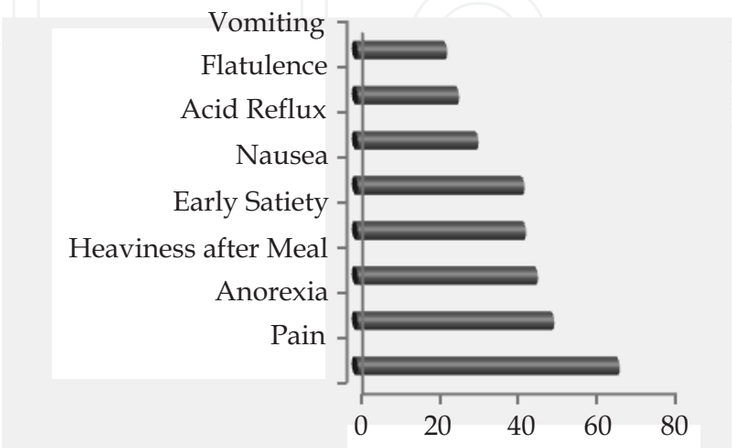

Fig. 7. Prevalence of patients reported symptoms in analysis 1

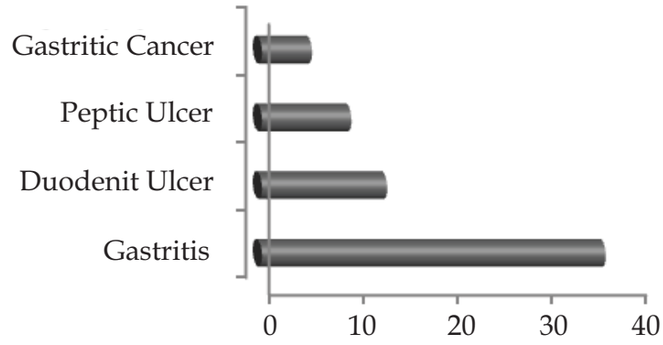

Fig. 8. Prevalence of clinical signs in analysis 1

8. Results

8.1 Analysis 1

Clinical features

Abdominal pain was present in 76% of patients (200/263), dyspepsia in 8%(20/263), vomiting in 6%(16/263), heartburn in 6%(17/263), and weakness 4%(10/263). The main endoscopic findings were 36% gastritis, 13% duodenal ulcer, 10% gastric ulcer, 9% esophagitis, and 5% gastric cancer (Figure 7, 8).

Culture, rapide urease test and PCR

H. pylori could be cultured from 84 of 263 (32%) patients, while a positive RUT or PCR band was observed in 54%(143 of 263) and 85%(223 of 263) of patients, respectively. Of the 84 patients with positive *H. pylori* culture 35/84 (41.7%) were males and 49/84 (58.3%) females. *H. pylori* was successfully cultured from 55 of 135 (41%) patients with non-ulcer dyspepsia and 29 of 62 (47%) with peptic ulcer (PU).

When the PCR was regarded as the "gold standard" of *H. pylori* identification, the sensitivity, specificity, PPV and NPV of RUT were 61%, 87%, 96% and 29% respectively. But for culture method were 36.77%, 95%, 97% and 21.22% respectively [59].

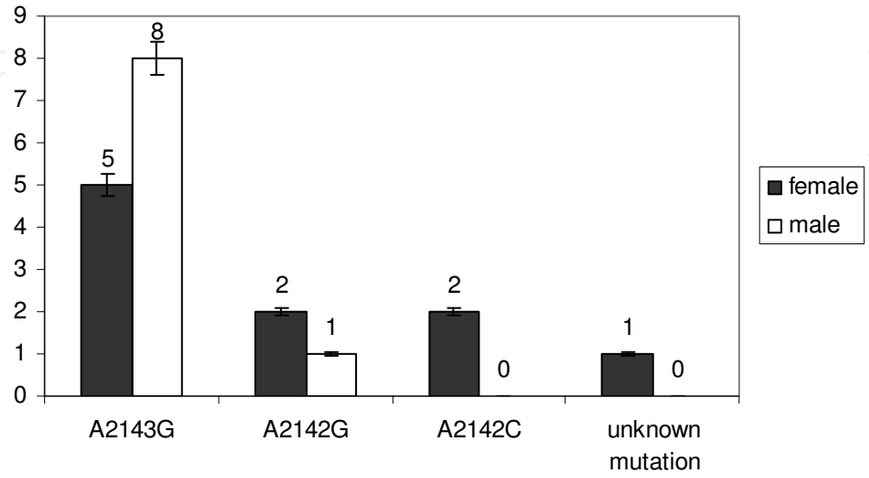

Fig. 9. Frequency of *23S rRNA* mutations in analysis 1 (n=19)

Prevalence of clarithromycin resistance

According to the agar dilution method, 19 of 84 (22.62%) cultured strains were resistant to clarithromycin. A 1.4kb fragment of the *23S rRNA* gene could be amplified in all 19 strains. Furthermore, *MboII* cuts PCR products were obtained from 3 and *BsaI* cuts another 13 amplified fragments of the resistant strains. In one strain, neither *MboII* nor *BsaI* was able to digest the amplicon. However, specially primed mismatched PCR yielded an amplicon, indicating that this strain had the A2142C mutation (Figure 9). No correlation was observed between the clarithromycin resistance, patient gender and clinical findings.

8.2 Analysis 2

Clinical features

164 patients with median age of 51.5 ranged from 15 to 88 years. Pain was present in 103 patient, nausea in 42, anorexia in 40, acid reflux in 33, heaviness after meal in 30, early satiety in 27, vomiting in 31 and flatulence in 42. The main endoscopic findings were 16 gastric ulcer, 3 gastric cancer, 55 non-ulcer disease, 50 gastric erosion, 34 nodularity, 37 gastritis and 3 duodenitis (Table 5).

Age group	Male	Female	Total
10-20	6 (3.66)	4 (2.44)	10 (6.10)
20-30	12 (7.32)	13 (7.93)	25 (15.24)
30-40	13 (7.93)	15 (9.15)	28 (17.07)
40-50	13 (7.93)	14 (8.54)	27 (16.46)
50-60	10 (6.10)	11 (6.71)	21 (12.80)
60-70	12 (7.32)	13 (7.93)	25 (15.25)
70-80	9 (5.49)	6 (3.66)	15 (9.15)
80-90	4 (2.44)	9 (5.49)	13 (7.93)
Total	79 (48.17)	85 (51.83)	164 (100)

Table 5. Distribution of *H. pylori* positive patients according to gender and age group (*n=164*)

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

RUT results showed that the 164 (82%) of the patients were *H. pylori*-positive. DNA samples derived from gastric biopsy samples of confirmed *H. pylori*-positive patients were positive by the diagnostic PCR assays for the *ureC* targets. The *ureC* PCR assay confirmed the presence of *H. pylori* in all of these 164 biopsy samples (100%) and generated the expected PCR product of 249 bp.

PCR-RFLP

Thirty-nine of 59 resistant strains detected by real-time PCR methods were detected by PCR-RFLP and were distributed as follows: 15 A2143G and 15 A2142G single mutation, 6 wild type/A2143G, one wild type/A2142G and 2 A2143G/A2142G genotypes (Table 6). *BsaI* cuts the PCR product of the wild-type sequence into two fragments of 1,000 and 400bp and that of the A2143G sequence into three fragments of 700, 400, and 300bp. *MboII* cuts the PCR product into two fragments of 700bp only when A2142G is present in the sequence (Figure 6). sensitivity and specificity of PCR-RFLP were 74.68% and 100% respectively when we PCR-RFLP was compared with real-time PCR.

Outcome	sample (%)	Genotype	
		Real-time-PCR	PCR-RFLP
Single genotype 143 (87.19)	105 (64.01)	Wild	Wild
	3 (1.83)	A2144G	Wild
	13(7.93)	A2143G	A2143G
	7(4.27)	A2143C	Wild
	15(9.15)	A2142G	A2142G
Heteroresistance 21 (12.81)	6(3.66)	A2143G/Wild	A2143G/Wild
	1(0.61)	A2143C/Wild	Wild
	1(0.61)	A2142G/Wild	A2142G/Wild
	1(0.61)	A2143G/A2143C/Wild	Wild
	1(0.61)	A2144G/A2143G/A2143C/ Wild	A2143G
	1(0.61)	A2143G/A2143C	A2143G
	1(0.61)	A2143G/A2143C	Wild
	1(0.61)	A2143G/A2142G	A2143G/A2142G
	1(0.61)	A2143C/A2142G	Wild
	1(0.61)	A2143G/A2143C/A2142G	A2143G/A2142G
	1(0.61)	A2143G/A2143C/A2142G	Wild
	5(3.05)	Not Determined	Wild

Table 6. Frequency of *H. pylori* genotypes determined by real-time PCR and PCR-RFLP methods in analysis 2 (n=164)

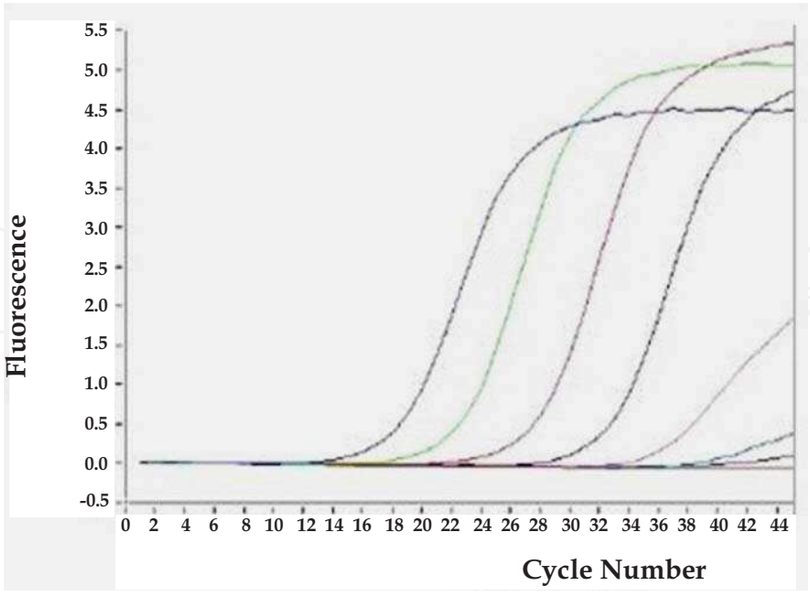

Fig. 10. Graph obtained with real-time PCR for the susceptible and resistant strains isolated from different patients. C_T for pure samples were less than 35 and C_T of mix samples were between 35 and 40. (All graphs obtained by different probes are similar)

Disease	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}	10^{12}	Total
Peptic Ulcer	1 (0.6)	0 (0)	7 (4.27)	4 (2.44)	2 (1.22)	1 (0.6)	0 (0)	0 (0)	0 (0)	15 (9.15)
Gastric Cancer	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.6)	0 (0)	2 (1.22)	3 (1.83)
Gastric Erosion	1 (0.6)	2 (1.22)	20 (12.19)	18 (10.98)	5 (3.05)	1 (0.6)	0 (0)	2 (1.22)	0 (0)	49 (29.88)
Nodularity	2 (1.22)	4 (2.44)	13 (7.93)	11 (6.71)	3 (1.83)	0 (0)	0 (0)	0 (0)	0 (0)	33 (20.12)
Gastritis	3 (1.83)	3 (1.83)	15 (9.15)	13 (7.93)	1 (0.6)	0 (0)	1 (0.6)	0 (0)	0 (0)	36 (21.95)
Duodenitis	0 (0)	1 (0.6)	1 (0.6)	1 (0.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.83)
Atrrophy	0 (0)	0 (0)	0 (0)	2 (1.22)	1 (0.6)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.83)
Duodenit Ulcer	0 (0)	0 (0)	13 (7.93)	8 (4.88)	1 (0.6)	0 (0)	0 (0)	0 (0)	0 (0)	22 (13.41)

Table 7. Endoscopic finding and frequency of bacterial count of *H. pylori* positive patients (*n=164*)

Symptoms	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}	10^{12}	Total
Pain	3 (1.83)	9 (5.49)	42 (25.61)	32 (19.51)	6 (3.66)	2 (1.22)	2 (1.22)	2 (1.22)	1 (0.6)	99 (60.36)
Nausea	3 (1.83)	1 (0.6)	20 (12.19)	13 (7.93)	3 (1.83)	0 (0)	0 (0)	1 (0.6)	1 (0.6)	42 (25.61)
Anorexia	3 (1.83)	3 (1.83)	18 (10.98)	14 (8.54)	0 (0)	1 (0.6)	0 (0)	0 (0)	0 (0)	39 (23.78)
Acid reflux	4 (2.44)	1 (0.6)	12 (7.32)	9 (5.49)	4 (2.44)	0 (0)	0 (0)	0 (0)	1 (0.6)	31 (18.90)
Heaviness after meal	1 (0.6)	3 (1.83)	9 (5.49)	12 (7.32)	3 (1.83)	0 (0)	1 (0.6)	0 (0)	0 (0)	29 (17.68)
Early satiety	2 (1.22)	1 (0.6)	13 (7.93)	8 (4.88)	1 (0.6)	0 (0)	1 (0.6)	0 (0)	0 (0)	26 (15.85)
Vomiting	3 (1.83)	2 (1.22)	12 (7.32)	10 (6.10)	1 (0.6)	0 (0)	0 (0)	0 (0)	1 (0.6)	29 (17.68)
Flatulence	3 (1.83)	4 (2.44)	19 (11.58)	11 (6.71)	1 (0.6)	0 (0)	1 (0.6)	0 (0)	1 (0.6)	40 (24.39)

Table 8. Interview data and bacterial count of *H. pylori* positive patients (*n=164*)

Determination of the cut-off for the C_T values

The detection of fluorescence was realized in the different channels of the rotor-gene thermocycler during 45 cycles of amplification. Among the 164 biopsy specimens, a detection of fluorescence was positive in one of the different channels in 143 (87.2%) cases. Sixteen biopsy specimens displayed more than one C_T values for mix genotypes. C_T values ranged from 21.4 to 43.6. C_T values less than 35 determined as pure sample DNA and C_T between 35 and 40 indicate as mix DNA sample. Detection of fluorescence over 40 cycles might be false positive (Figure 10).

Development of TaqMan real-time PCR

Concerning amplification of *H. pylori* DNA obtained from a pure culture, linearity was achieved over a 5-log range of input DNA amounts, from 5×10^2 to 5×10^7 bacteria, with 500 bacteria corresponding to 1000 *23S rRNA* gene copies given that there are two gene copies in the genome of *H. pylori*. PCR was also positive for 40 bacteria or 80 copies of the *23S rRNA*

gene fragment. The amplification efficiencies obtained with DNA prepared from gastric biopsy specimens were identical to those obtained with DNA samples prepared from bacterial colonies. Thus, the sensitivity of our assay, for DNA samples prepared either from cultures or from gastric biopsy specimens, could quantitatively reach 400 bacteria or 800 copies of the amplicon and qualitatively reach 40 bacteria or 80 copies. The reproducibility of our assay was evaluated with 10-fold serial dilutions of purified *H. pylori* DNA, with no significant difference between runs.

Real-time PCR

Bacterial clarithromycin resistance was assessed on 164 consecutive, *H. pylori*-positive patients. Overall, a clarithromycin susceptible was detected in 105 (64.02%) patients and clarithromycin resistance was detected in 59 (35.98%) which were identified as 4 (6.78%) A2144G, 26 (44.07%) A2143G, 15 (25.42%) A2143C and 20 (33.9%) A2142G point mutations. Purely resistant strains were detected in 38 (64.41%), while a mixture of resistant and susceptible (heteroresistant) bacterial strains were found in the remaining 16 (27.12%) cases. Genotype of 5 (8.47%) strains were not detected (Table 6) [60].

Quantification of bacterial density

Quantification of different genotypes was directly performed on 159 DNA samples with defined genotype by using TaqMan real-time-PCR assay. The bacterial density by this technique could be evaluated for 159 *H. pylori*-positive patients and ranged from 1.53×10^4 to 5.89×10^{12}. In order to evaluate relationship between bacterial concentration and point mutations, samples divided in 9 groups between 1×10^4 to 6×10^{12}, including 10^4, 10^5, 10^6, 10^7, 10^8, 10^9,10^{10}, 10^{11} and 10^{12} groups had 6, 11, 87, 59, 9, 2, 2, 2 and 2 samples respectively (Table 7 and 8).

Bacterial count	Wild type	A2144G	A2143G	A2143C	A2142G
10^4	1	0	2	2	1
10^5	7	0	2	1	1
10^6	39	4	18	12	14
10^7	51	0	4	0	4
10^8	9	0	0	0	0
10^9	2	0	0	0	0
10^{10}	2	0	0	0	0
10^{11}	2	0	0	0	0
10^{12}	2	0	0	0	0
Total	105	4	26	15	20

Table 9. Distribution of 5 different genotypes based on their concentrations

Statistical analysis

Chi-square analysis revealed more relationship between gastritis and age group 30-40 (p=0.007), NUD and 40-50 (p=0.001), early satiety and 10-20 (p=0.05), flatulence and 10-20

(p=0.038), vomiting and 20-30 (p=0.001), pain and 50-60 (p=0.003), heaviness after meal and 50-60 (p=0.018), early satiety and 60-70 (p=0.05).
Also statistical analysis revealed more relationship between wild type and 10^6 (p=0.00) and 10^7 (p=0.00), A2144G and 10^6 (p=0.033), A2143G and 10^6 (p=0.005), A2143C and 10^6 (p=0.005) and A2142G and 10^6 (p=0.015) (Table 9) [61].
Also, results showed relation ships between Ages 10-20 and early satiety (p=0.05) and flatulence (p=0.038), 20-30 and vomiting (p=0.001), 50-60 and pain (p=0.003) and heaviness after meal (p=0.018) and 50-60 and early satiety (p=0.05).

9. Conclusion

In conclusion, we have developed a TaqMan real-time PCR assay that permits accurate, fast, and cost-effective detection of *H. pylori* directly from gastric biopsy specimens as well as detection of clarithromycin resistance. This PCR technique is a good candidate for automated real-time PCR methods allowing simple and rapid detection of *H. pylori* and its resistance to clarithromycin by clinical laboratories which do not practice *H. pylori* culture. Identification of sensitive and resistant strains by using PCR-RFLP method was based on the determination of resistant strains. Therefore, other resistant strains, which mutations cannot be identified by PCR-RFLP method, are considered as sensitive. But, Identification of sensitive and resistant strains by using real-time PCR method was based on the determination of sensitive strains. In the first step, determination of sensitive, resistance and mix of sensitive and resistance strains were done and in the next step, determination of mutations types in resistance and mix strains were done, that leading to accurate diagnosis of the resistant and sensitive strains.
Based on the patterns of competitive growth as well as the individual growth of different clarithromycin resistance mutant strains, Wang and colleagues conclude that the order of preference of competitive accumulation is A2142G A2143G A2142C A2143C (A2142T). If the same is true in vivo, once an A-to-G transient mutation occurs (spontaneously or drug induced), the other types of mutation that exist in the same environment, if any, are likely to be overgrown after a period of time. A-to-C or A-to-T mutants could be isolated only when an A-to-G mutant has not appeared at that particular gastric niche. Their results provide a rational explanation for the mutation pattern observed in clinical isolates [13]. These results are confirmed by Van der Ende and coworkers and discussed that the growth rates of *H. pylori* isolates with the A21423G, A21423C, or A21433G mutation did not differ from that of the wild type, but *H. pylori* isolates with other *23S rDNA* mutations grew more slowly [11].
Based on two opinions, we used real-time PCR for simultaneous identification of *H. pylori*, Clarithromycin resistance point mutations and direct quantification of gastric mucosal density in order to answer to this question; why some clarithromycin resistance point mutations are more prevalent than other mutations. At first; Wang described an additional possible mechanism yet to be identified, by which the A-to-G mutations are preferentially produced in *H. pylori*, may also contribute to the observed predominance of A-to-G mutations [13]. Second; Van der Ende [11] described that; If only a single colony from the primary *H. pylori* populations is used to test for clarithromycin susceptibility, the results can be misinterpreted. Assessment of *23S rRNA* mutations in *H. pylori* directly from biopsy specimens by molecular biological techniques has the advantage that infection with a mixed *H. pylori* population is easily detected. In addition, knowledge of the type of *23S rRNA*

mutation may be important since clarithromycin MICs are associated with the type of *23S rRNA* mutation in *H. pylori* [11].

Several studies which done by Pina [34], Marais [28] and Russmann [36] in Europe revealed that A2144G, A2143G and A2143C were more prevalent in Europe respectively. However, the current study showed that A2143G, A2142G, A2143C and A2144G were more prevalent mutation in Iran respectively. Our data showed that wild type strains more related to 10^6 to 10^7 bacteria in gastric biopsy specimens of patients. Also, strains with clarithromycin resistance mutation assessed in this study are significantly related to 10^6 in gastric biopsy which are equal to density of wild type strain. This data revealed that these mutated strains has a same growth rate to wild type, preferentially produced in populations and selected by natural selection force and they can exist and distributed in *H. pylori* population

10. Acknowledgment

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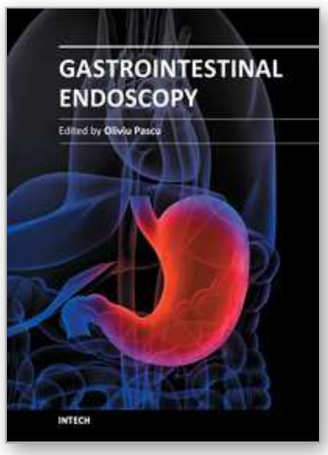

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Endoscopy has had a major impact in the development of modern gastroenterology. By using different data it provided a better understanding of pathogenic mechanisms, described new entities and changed diagnostic and therapeutic strategies. Meanwhile, taking advantage of many technical advances, endoscopy has had a developed spectacularly. Video-endoscopes, magnification, confocal and narrow-band imaging endoscopes, endoscopic ultrasounds and enteroscopes emerged. Moreover, endoscopy has surpassed its function as an examination tool and it became a rapid and efficient therapeutic tool of low invasiveness. InTech Open Access Publisher selected several known names from all continents and countries with different levels of development. Multiple specific points of view, with respect to different origins of the authors were presented together with various topics regarding diagnostic or therapeutic endoscopy. This book represents a valuable tool for formation and continuous medical education in endoscopy considering the performances or technical possibilities in different parts of the world.

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