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Real-time PCR for *Helicobacter pylori* quantification and detection of clarithromycin resistance in gastric tissue from patients with gastrointestinal disorders

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Abstract

Helicobacter pylori gram-negative bacteria commonly infect the human gastrointestinal (GI) tract and are readily diagnosed by endoscopy. *H. pylori* infection causes a broad range of host symptoms from discomfort to significant GI disorders (GIDs). Severity of the clinical manifestations depends mainly upon bacterial load. In this cross-sectional study, we investigated the affects of *23S rRNA* point mutations on *H. pylori* count in naturally infected human GI tissues. Two-hundred *H. pylori* patients with suspected GIDs were evaluated to determine bacteria concentration and presence of four known *23S rRNA* point mutations, causing clarithromycin resistance. Gastric biopsy specimens were examined by rapid urease test and *16S rRNA*-targeted PCR to identify *H. pylori*; then bacterial load was quantified by real-time PCR targeting wild type and known *23S rRNA* mutations. Eighty-two percent of the samples were confirmed as *H. pylori*-positive, having 10^4–10^{12} colony-forming units (CFU)/ml. The 10^6 load was most strongly associated with peptidyltransferase point mutations of the *23S rRNA* gene A2144G ($p = 0.033$), A2143G ($p = 0.005$), A2143C ($p = 0.005$), and A2142G ($p = 0.015$). Thus, our findings indicated that dominant *23S rRNA* mutated *H. pylori* strains have the same growth rate as the wild type in a gastric environment.

Keywords: *Helicobacter pylori*; Bacterial load; *23S rRNA*; Point mutations; Real-time PCR

1. Introduction

Helicobacter pylori represents the most commonly diagnosed chronic gastric bacterial infection in the world (Oleastro et al., 2003) and is a major cause of gastritis, peptic ulcer disease and gastric cancer (Kargar et al., 2010). Severity of the clinical manifestations of this infection is associated with bacterial load (Kusters et al., 2006). The preferred eradication therapy is a combination triple antibiotic consisting of metronidazole, omeprazole and clarithromycin, which boasts a 90% success rate. However, the efficacy of this therapeutic strategy drops to between 0 and 50% when clarithromycin-resistant *H. pylori* strains are present (Chisholm et al., 2001; Oleastro et al., 2003).

Clarithromycin acts to halt bacterial growth by physically interacting with the 23S rRNA component of the bacterial ribosome, a key component in the protein synthesis machinery. Some *H. pylori* strains have evolved resistance to clarithromycin by mutation of the genetic sequence in the targeted binding site. Specifically, single spontaneous point mutations in the peptidyltransferase-coding region of the *23S rRNA* gene have been implicated in generation of the resistant phenotype (Kargar et al., 2011a; Lascols et al., 2003). The four most often observed mutations are: A2144G, A2143G, A2142G and A2143C. Other mutations, such as A2142C, A2115G, G2141A, A2142T and T2717C, have been reported but to

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a much lesser extent (Lascols et al., 2003; Van Der Ende et al., 2001).

Determination of *H. pylori* density in gastric mucosa biopsies is used to stratify patients for treatment and determine prognosis; this is historically carried out in clinic by bacterial culture and histological analysis of infected tissues. However, both of these approaches are time-consuming and the histological observations are limited by their subjective nature (Lascols et al., 2003). More recently, PCR-based diagnostics have been introduced, including amplification of either the *ureC* or *16S rRNA* bacterial genes.

In this study we sought to develop a more quantitative real-time PCR-based diagnostic strategy based on the *23S rRNA* gene. The advantages of this approach were expected to be threefold. First, by using whole samples of DNA isolated directly from gastric biopsy tissues of infected individuals, the presence of multiple *H. pylori* strains would be simultaneously detectable from a single sample. Second, the genomic sequence of the *H. pylori* strains would reveal the presence or absence of mutations associated with the clarithromycin-resistant phenotype. Third, the obtained sequences would be applicable to comparative analysis studies to determine the relationship with associated bacterial properties (growth) and the prevalence of certain point mutations.

2. Material and methods

2.1. Study design

Gastric biopsy specimens were obtained from 200 patients from the Chaharmahal and Bakhtiari province who had been referred to local hospitals for upper gastrointestinal tract endoscopies to diagnose dyspeptic symptoms between June and November 2009. All patients provided written informed consent prior to endoscopy. Two antral samples and one corpus sample were taken from each patient. *H. pylori* positivity was initially determined by the standard rapid urease test (RUT) using the Gastro Urease kit (Bahar-afshan, Co., Tehran, Iran). All of the samples were transported to the laboratory under a strict cold chain and stored at −70 °C until further investigation. DNA was isolated from each tissue by using the DNA extraction kit DNP™ from CinnaGen (Tehran, Iran) according to the manufacturer's instructions. DNA was immediately subjected to molecular analysis, including conventional PCR (for *H. pylori* diagnostic) and real-time PCR (for *H. pylori* diagnostic, quantification and identification of clarithromycin-resistant genotype).

2.2. Confirmation of H. pylori in gastric biopsy specimens

PCR targeting the *16S rRNA* gene was carried out to confirm *H. pylori* in each specimen. The previously designed and verified primer pair HP-1 and HP-2 was synthesized for this purpose (Kargar et al., 2011b) (Table 1). PCR amplification of genomic DNA was performed in a reaction mixture of 25 µl which contained 2 µl biopsy-isolated DNA template, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs) mixture, 0.2 mM of each primer and 1 U of *Taq* DNA polymerase (CinnaGen, Co., Tehran, Iran). The following thermal-cycling conditions were used: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The resulting Hp16S fragment (expected size of 109 base pairs) was visualized after electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Table 1
Oligonucleotide sequences used in this study.

Primer/probe	Sequence (5′ → 3′)	Target
HP-1	CTGGAGAGACTAAGCCCTCC	*16S rRNA*
HP-2	ATTACTGACGCTGATTGTGC	
HP23S-1	CCACAGCGATGTGGTCTCAG	*23S rRNA*
HP23S-2	CTCCATAAGAGCCAAAGCCC	
Pwt	Cy5-GGGGTCTTTCCGTCT-BHQ2	Wild type
P44G	TAMRA-GGTCCTTCCGTCTTG-Dabcyl	A2144G
P43G	TET-GGTCTCTCCGTCTTG-Dabcyl	A2143G
P43C	HEX-GGTCTGTCCGTCTTG-Dabcyl	A2143C
P42G	FAM-GGTCTTCCCGTCTTG-Dabcyl	A2142G

2.3. Identification of the clarithromycin resistance genomic mutation

*Taq*Man real-time PCR was performed with the primer pair HP23S-1 and HP23S-2 and either modified probes Pwt, P44G, P43G and P43C (previously reported by Pina et al.,) or newly designed probe P42G (according to the *23S rRNA* gene sequence from GenBank accession no. U27270) in order to identify wild type, A2144G, A2143G, A2143C and A2142G genotypes, respectively (Table 1).

Real-time PCR analysis was performed on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia). Briefly, the 25 µl PCR mixture containing 2 µl of extracted DNA, 200 mM dNTPs (CinnaGen, Co., Tehran, Iran), 0.2 mM (each) primer HP23S-1 and HP23S-2, 0.1 mM probe Pwt, 0.2 mM probe P44G, 0.1 mM probe P43G, 0.1 mM probe P43C and 0.75 mM probe P42G (Bioneer, Daejeon, Korea), 1.5 mM $MgCl_2$, and 1.5 U of *Taq* polymerase in PCR buffer (CinnaGen, Co., Tehran, Iran), was denatured at 95 °C for 5 min; amplification was carried out in 45 cycles of 95 °C for 30 s and 58 °C for 40 s. The fluorescence reading for each sample was taken at the annealing step on different channels. All samples were run in duplicate and positive and negative controls were included in each assay. Data were analyzed with instrument compliant software (*ver1.7*; Corbett Research). In order to test the specificity of the primers, purified DNA from non-*H. pylori* bacterial strains was used as template. The cycle threshold (C_q) value was calculated for each channel as the number of cycles at which fluorescence exceeded the threshold limit, which was set at the top of the second derivative fluorescence curve and expressed as fractional cycle numbers.

2.4. Determination of bacterial load by real-time PCR

Standard curves were generated by PCR of serial dilutions. After DNA extraction, one 10-fold serial dilution of *H. pylori*