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Prevalence of *Helicobacter pylori* in Symptomatic Patients and Detection of Clarithromycin Resistance Using Melting Curve Analysis

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ABSTRACT

Background: Clarithromycin is often a component of combination therapies for *Helicobacter pylori* eradication; however, increases in resistance rates have decreased the success of the treatment.

Objective: This study was designed to determine the prevalence of *H pylori* infection in symptomatic patients and to detect clarithromycin resistance rates using melting curve analysis.

Methods: Patients scheduled for upper endoscopy at the Endoscopy Unit of the Department of Gastroenterology, Duzce University, Medical Faculty Hospital, Konuralp/Duzce, Turkey, were assessed for enrollment in the study. Two pairs of gastric biopsy specimens (antrum and corpus) were obtained from each study patient. Histopathologic examination, rapid urease test, culture, and polymerase chain reaction (PCR) of the specimens were used to identify *H pylori* infection. Clarithromycin resistance was detected using melting curve analysis.

Results: Seventy-five patients (41 women, 34 men; mean [SD] age, 42.6 [14.5] years [range, 17–70 years]) were included in the study. Using histopathology and rapid urease test, *H pylori* was detected in 40 (53.3%) of the 75 specimens. *H pylori* was detected using PCR in 40 (53.3%) specimens and by culture in 10 (13.3%) specimens. The specificity and sensitivity of PCR and culture were interpreted by comparing them with the results of histopathologic examination and urease tests. The specificity and sensitivity of PCR were 68.6% and 72.5%, respectively, and the specificity and sensitivity of culture were 97.1% and 22.5%, respectively. Of the 40 isolates, 21 (52.5%) were susceptible to clarithromycin, 12 (30.0%) were resistant, and a mixed susceptibility

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pattern was detected in 7 (17.5%) specimens. *H pylori* isolates from 19 (79.2%) of the 24 patients who had formerly used clarithromycin showed clarithromycin resistance.

Conclusions: The prevalence of *H pylori* infection was 53.3% for the symptomatic patients in this study, and 47.5% of the isolates showed clarithromycin resistance using melting curve analysis. The PCR-based system used in this study was accurate for the detection of *H pylori* infection as well as clarithromycin susceptibility testing directly in biopsy specimens. (*Curr Ther Res Clin Exp*. 2007;68: 151–160) Copyright © 2007 Excerpta Medica, Inc.

Key words: *Helicobacter pylori*, clarithromycin resistance, melting curve analysis, PCR.

INTRODUCTION

Helicobacter pylori bacteria is a frequent cause of chronic infection worldwide, especially in developing countries.[1] This pathogen causes both intestinal and extra-intestinal complaints. Eradication regimens for *H pylori* usually contain 2 antibiotics, and the macrolide drug clarithromycin is often one of the drugs used. However, resistance to clarithromycin has increased, leading to decreased treatment success. A high rate of *H pylori* resistance to clarithromycin has been observed. Previous macrolide use has been reported to be an important risk factor for clarithromycin resistance.[2–4]

Clarithromycin resistance of *H pylori* is caused by point mutations of the 23 soluble RNA gene, the main component of the 50S ribosomal subunit, mostly at positions 2142 and 2143 (2142A→G, 2142A→C, 2142A→T; 2143A→G, 2143A→C) in the peptidyltransferase region of the variable domain. These point mutations prevent the drug from binding with *H pylori*.[5] The melting curve analysis method is a real-time polymerase chain reaction (PCR) assay that can be used to detect clarithromycin resistance. This method allows rapid isolation of *H pylori* DNA directly from gastric biopsies as well as detection of the most common point mutations that confer resistance to clarithromycin.[6]

The aim of this study was to determine the prevalence of *H pylori* infection in symptomatic patients undergoing endoscopy and to determine the clarithromycin resistance rate using the melting curve analysis method.

PATIENTS AND METHODS

Patients

Patients coming to the Endoscopy Unit of the Department of Gastroenterology, Duzce University, Medical Faculty Hospital, Konuralp/Duzce, Turkey, for upper endoscopy were asked to participate in the study. Patient inclusion criteria were a history of upper gastrointestinal tract symptoms that were suggestive of dyspepsia and the possibility of previous ulceration. Patients who had medical contraindications for the biopsy procedure and those who used antimicrobials

or proton pump inhibitor drugs within 2 weeks before the study were excluded. Previous use of clarithromycin was noted. The ethics committee of the hospital approved the study. All patients were required to provide written informed consent prior to study participation.

Gastric Biopsy Specimens

Every patient underwent upper endoscopy during which a total of 4 gastric biopsy specimens were taken from the antrum (2) and the corpus (2). One pair of samples (1 antrum and 1 corpus) was subjected to histopathologic examination and stained with hematoxylin-eosin to identify *H pylori* using light microscopy. The other pair of samples was placed in 2 mL of 0.85% normal sterile saline, immediately transported on ice to the laboratory, and processed within 30 minutes for bacteriologic examination. All samples were subjected to the PCR procedure, a rapid urease test, and cultured.

Culture and Identification of *Helicobacter pylori*

Biopsy specimens were cultured on Columbia agar containing 7% laked horse blood (Invitrogen, Auckland, New Zealand) with Dent *H pylori* antibiotic supplement (Oxoid, Hampshire, United Kingdom) and were incubated at 37°C for up to 10 days in anaerobic jars (without catalyst) with CampyGen packs (Oxoid). *H pylori* colonies were identified by their characteristic morphology, positive urease test, and appearance of cells recovered after Gram staining.[7] *H pylori* NCTC 11637 was used as a positive control.

Helicobacter pylori Detection in Gastric Biopsy Specimens

For each patient, a pair of biopsy samples was stored at –40°C up to the day of the PCR, which was performed at Metis Biotechnology Laboratory (Ankara, Turkey). DNA was extracted from homogenized biopsy samples using the phenol-chloroform extraction method.[8] *H pylori* DNA was screened using a nested PCR amplification method for a portion of the 23S ribosomal RNA (rRNA) gene. Five microliters of extracted DNA was added to each PCR amplification reaction.

The reaction was performed using outer primers whose nucleotide sequence was derived from a known sequence of the 23S rRNA gene: Hp 23 OF 1pp (the outer forward primer): 5′-AACGGTCCTAAGGTAGCGAA-3′; CRL 2 (the outer reverse primer): 5′-ACACTCAACTTGCGATTCC-3′, which generates a 408-bp product; CRLF 1 (the inner forward primer): 5′-ATGAATGGCGTAACGAGAT-3′; and Hp 23R (the inner reverse primer): 5′-GTGCTAAGTTGTAGTAAAGGT-3′, which generates a 126-bp product.

The PCR amplification reaction mixture (50 µL) contained the following: nuclease-free water 36.3 µL, 10× PCR buffer 5 µL, deoxyribonucleoside triphosphates 0.5 µL (10 mM), Taq polimeráz 0.2 µL (5 U/µL, Sigma, Steinheim, Germany), primers 0.5 µL (100 pmol/µL), magnesium 2.5 mM, and a 5-µL DNA sample. The PCR cycle (Techne, Cambridge, United Kingdom) conditions were

as follows: 1 cycle at 94°C for 5 minutes; 30 cycles each at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute; 1 cycle 72°C for 5 minutes. Negative and positive controls were *Campylobacter jejuni* ATCC 33560 and *H pylori* NCTC 11637, respectively.

The PCR products were analyzed using gel electrophoresis with 2% (w/v) agarose stained in ethidium bromide 0.5 mg/L and examined by ultraviolet transillumination.

Real-time PCR amplification and melting curve analysis of the *H pylori* 23S rRNA gene were performed. A real-time PCR hybridization assay was used to detect point mutations conferring resistance to clarithromycin on the outer PCR products obtained from gastric biopsies that were found to be positive for *H pylori*. The method included amplification of a fragment of the *H pylori* 23S rRNA gene coupled with simultaneous detection of the product by probe hybridization and analysis of the melting curve using real-time PCR.

Real-time PCR assay was used to detect clarithromycin resistance-associated point mutations on the 23S rRNA gene using primers, probes, and reaction conditions described by Chisholm et al.[9] After amplification of the 96-bp region, clarithromycin resistance-related mutations of 2143A→C, 2143A→G, and 2144A→G were analyzed using melting curve analysis with LightCycler software version 3.5.3 (LightCycler, Mannheim, Germany). Melting point peaks of 82°C to 86°C found *H pylori* in the sample. Probe melting point peaks of positive samples were analyzed using the same method. Sensitive strains, 2143A→C, 2143A→G, 2144A→G, gave peaks of ~65°C, ~60°C, and ~55°C, respectively.

Statistical Analysis

Histopathologic examination and urease tests were considered to be the gold standard. The results of PCR and culture were interpreted by comparing them with the results of histopathologic examination and urease tests. The sensitivity and specificity of the tests were evaluated statistically. Statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, Illinois). StatCalc 6.0 (Epi Info™, Centers for Disease Control and Prevention, Atlanta, Georgia) was used to calculate the sample size. The sample comprised 78 patients, given a population of 312,000 people, a 90% prevalence of *H pylori* infection in symptomatic people, and a 80% confidence level.

RESULTS

A total of 78 patients were approached to participate in the study. Three patients were excluded because they were receiving antimicrobial therapy for respiratory tract infections. Thus, 75 patients (41 females, 34 males; mean [SD] age, 42.6 [14.5] years [range, 17–70 years]) were included in the study. Thirty-two (42.7%) of the patients had a history of clarithromycin use.

Of the patients studied, 40 (53.3%) were identified as having *H pylori* infection by histopathologic examination and rapid urease test, whereas 35 (46.7%) showed

no evidence of *H pylori* infection. Of the 40 patients with *H pylori* infection, 24 (60.0%) stated they had previously used clarithromycin.

When the paired specimens for the 40 patients with *H pylori* infection were analyzed by PCR and culture, *H pylori* was detected by PCR in 40 (53.3%) specimens and by culture in 10 (13.3%) (**Table**). PCR products of the anticipated size (294 bp) were obtained from biopsy specimens from both the antrum and corpus of all positive samples.

Compared with histopathology and rapid urease test for the detection of *H pylori*, the specificity and sensitivity of PCR were 68.6% and 72.5%, respectively, and the specificity and sensitivity of culture were 97.1% and 22.5%, respectively.

The clarithromycin resistance rates of *H pylori* in our study (n = 40), as measured using melting curve analysis, were as follows: 21 (52.5%) susceptible, 12 (30.0%) resistant, and 7 (17.5%) mixed susceptibility (resistant + susceptible). Among the patients who had previously used clarithromycin, 79.2% (19/24) had clarithromycin-resistant strains.

DISCUSSION

H pylori colonizes the mucosa of the human stomach, where it can establish long-term infection that is associated with acute or chronic gastric inflammation that may progress to peptic ulcer disease, atrophic gastritis with intestinal metaplasia, or gastric cancer.[10,11] A variety of clinical outcomes of *H pylori* infection are associated with both host factors and bacterial virulence factors.[10]

In developing countries, 70% to 90% of the population have been found to have *H pylori* infection.[11] In developed countries, the prevalence of infection is lower. Transmission can occur by iatrogenic, fecal-oral, and oral-oral routes. *H pylori* is able to colonize and persist in a unique biological niche within the gastric lumen.[11]

Several diagnostic assays for *H pylori* detection are currently available. Invasive methods requiring gastric endoscopy include rapid urease testing, culture, histopathology, and molecular diagnostics. Noninvasive approaches include fecal antigen detection, serologic testing for immunoglobulin G, and urea breath testing.[12] The noninvasive methods have recently gained in impor-

Table. The results of histopathology + rapid urease test, polymerase chain reaction (PCR), and culture in symptomatic patients undergoing upper endoscopy (N = 75). Values are no. (%).

Test	Positive	Negative
Histopathology + rapid urease test	40 (53.3)	35 (46.7)
PCR	40 (53.3)	35 (46.7)
Culture	10 (13.3)	65 (86.7)

tance[13]; however, no information about antibiotic resistance has been obtained using these tests.

Histopathology is the gold standard for diagnosing *H pylori* infection and is generally more sensitive than culture.[13] Although histopathology allows direct visualization of the organism and the extent and nature of tissue involvement, it is associated with several problems. If gastritis is patchy and a biopsy is performed on a non-infected area or if only a small number of organisms are present, the sensitivity declines. Moreover, the examination requires 1 to 3 days to complete. Culture permits determination of antimicrobial susceptibilities and pathogenic features of isolates. The disadvantages of culture include the special conditions required to transport the specimen, the use of complicated media with particular maintenance conditions, the need for specific incubation conditions, and the length of time necessary to obtain a result.[9] Urease detection is rapid, but increased sensitivity requires longer incubation and bacterial overgrowth may cause false-positive results.[14] The PCR method allows rapid isolation of *H pylori* directly from gastric biopsies and detects the most common point mutations that confer resistance to clarithromycin.[6] PCR is also the most accurate method among the biopsy-based tests used to detect *H pylori* infection in patients with bleeding peptic ulcers, although blood may reduce the sensitivity of all biopsy-based tests.[15] In clinical practice, the simple, rapid PCR assay used in this study might be of great value, especially when the emerging problem of clarithromycin-resistant strains is being considered, and may have a significant impact on patient management.

Our analysis revealed that the specificity and sensitivity of PCR were 68.6% and 72.5%, respectively, and the specificity and sensitivity of culture were 97.1% and 22.5%, respectively.

Eradication therapy is recommended for patients with peptic ulcer disease. The first-line regimen usually consists of a 2-drug therapy, with clarithromycin being one of the most widely used components in these treatments.[6] The MIC of clarithromycin is low and is relatively unaffected by lowering the pH. The drug reaches a high concentration in gastric mucosa with a high degree of binding to *H pylori* ribosomes.[16]

Resistance of *H pylori* to clarithromycin is mainly due to an adenine-to-guanine transition at positions 2142 and 2143 and to an adenine-to-cytosine transversion at position 2142, which are included in the peptidyltransferase loop of the 23S rRNA gene.[17] The most frequently (98%) observed mutations are 2142A→ and 2143A→G, with the 2142A→C mutation being much rarer (1.6%).[18] Other mutations (2115A→G, 2141G→A, and 2717T→C) have been described but appear to be infrequent.[19,20]

Several methods are available to test *H pylori* drug susceptibility, including the disc test, the E test, the microplate method, the agar plate dilution method, and PCR-based techniques. The E test is the standard method used in European countries and the United States, while no standard method of *H pylori* drug susceptibility testing has been established in Japan.[21] Several PCR-based methods

(eg, PCR-restriction fragment length polymorphism, PCR-DNA-enzyme immunoassay, reverse hybridization line probe assay, and real-time PCR methods combined with melting curve analysis by biprobes and hyprobes) have been performed with cultured strains or biopsies to determine susceptibility to clarithromycin.[22] Some of the advantages of real-time PCR are rapidity, low contamination rate because of a closed-tube system, and the possibility of accurate quantification of the DNA target.[23] Melting curve analysis is a real-time PCR assay that can be used directly on gastric biopsies to detect the most common point mutations occurring in the 23S rRNA gene of *H pylori* that confer resistance to clarithromycin. The entire procedure requires only 2 hours from the time specimens are received, from the isolation of DNA from the biopsies to the detection of the mutations.[6] The identification of specific point mutations in the 23S rRNA gene has enabled the development of molecular tests that allow determination of clarithromycin resistance directly from the biopsies. The 3 most common mutations (2142A→G, 2143A→G, and 2142A→C) are detectable directly from gastric biopsy specimens, thus avoiding the delay associated with culture-based susceptibility testing.[9] In this study, melting curve analysis allowed us to rapidly determine the susceptibilities of all gastric biopsy isolates, overcoming the delays associated with conventional culture methods for *H pylori* identification and susceptibility testing.

The frequency of antibiotic resistance has been found to vary widely by geographical regions and subgroups within study populations.[14,24] The global primary resistance rate for clarithromycin was found to be 9.9%. However, notable differences were observed when clarithromycin resistance rates were broken down by regions in Europe (ie, 4.2% in Northern Europe, 9.3% in Central/Eastern Europe, and 18% in Southern Europe).[3,25] A systematic review of the studies performed in Canada before the year 2000 estimated resistance to be >4%. However, resistance is reported as 10% to 15% in the United States, regardless of region. In the Middle East, prevalence rates of 5.4% in Israel and 17% in Iran have been reported. In the Far East, the prevalence is higher in Japan (11%–12%) than in Hong Kong (4.5%) and Korea (5%–6%).[25]

The prevalence of primary- and acquired-clarithromycin resistance is increasing worldwide, jeopardizing the success of these treatments.[6] For example, the resistance rates of *H pylori* to clarithromycin in Beijing were 10.0% (5/50) in 1999 to 2000 and 18.3% (20/109) in 2001 to 2002 with increasing resistance rates.[26] In Japan, use of clarithromycin increased 4-fold between 1993 and 2000, which resulted in a similar 4-fold increase in the resistance rate.[27]

In Turkey, resistance rates of *H pylori* differ, ranging from 5% to 24.2%.[28–30] In these studies, resistance was detected using microbiological techniques, such as the E test. Baglan et al[16] detected clarithromycin resistance in 27.6% of isolates using PCR Restriction Fragment Length Polymorphism, a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. Our results, with 30.0% clarithromycin resistance and 17.5% mixed susceptibility, support those findings. The increase in the preva-

lence of resistant *H pylori* isolates as a result of widespread oral therapy with macrolides for presumptive respiratory or gastrointestinal tract infections has been reported.[2–4,17] Our resistance rates might be related to the frequent consumption of clarithromycin, as 60.0% of the patients with *H pylori* infection stated they had previously used the drug and resistance was detected in 79.2% of these patients.

CONCLUSION

The prevalence of *H pylori* infection was 53.3% for patients who had met the criteria for endoscopy, and 47.5% of the isolates showed clarithromycin resistance using the melting curve analysis method. Sixty percent of the patients with *H pylori* infection had a history of previous clarithromycin use. In evaluating clarithromycin resistance, the PCR-based system used in this study allowed the accurate detection of *H pylori* infection and clarithromycin susceptibility testing directly in biopsy samples.

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