

Comparison of FISH, RFLP and agar dilution methods for testing clarithromycin resistance of *Helicobacter pylori*

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ABSTRACT

Background/Aims: Clarithromycin resistance is an important factor of *Helicobacter pylori* (*H. pylori*) eradication failure in adults and children. There are some tests to determine resistance such as restriction fragment length polymorphism (RFLP), fluorescence in situ hybridisation (FISH), PCR and (culture) agar dilution. Clarithromycin resistance is reported between 16.8%-48.2% in Turkey using PCR, 18% in Japan using RFLP. The aim of the study is to compare the efficacy of FISH, RFLP and culture.

Materials and Methods: Patients with gastric complaint underwent endoscopic examination. *H. pylori* status of all patients was tested with urea breath test. Gastric biopsy samples obtained from adult patients and children were studied. Each tissue was analysed with FISH, PCR-RFLP and agar dilution.

Results: A total 100 patients were positive by UBT and histology for *H. pylori*. Tissues from 89 adults and 11 children were evaluated. According to FISH and RFLP clarithromycin resistance was 26% and 16% respectively. Among 100 patients *H. pylori* was cultured in 52 tissue samples, among these samples 7 were resistant to clarithromycin. There was strong correlation between the results of FISH and RFLP; RFLP and culture; and FISH and culture.

Conclusion: There is a high ratio of clarithromycin resistance in the studied population. All 3 tests are valuable, but FISH seems to be more sensitive among these tests. We suggest FISH should be used for detecting clarithromycin resistance among *H. pylori* infected patients before eradication therapy.

Keywords: *H. pylori*, clarithromycin resistance, FISH, PCR, RFLP

INTRODUCTION

Since the discovery of *Helicobacter pylori* (*H. pylori*) in adult patients by Warren and Marshall much has been learned (1). Today *H. pylori* infection is known as the cause of gastritis, peptic ulcer disease, gastric cancer and gastric mucosa associated lymphoma (2). Moreover it is suggested that this infection may lead to an increased risk of developing precancerous gastric conditions, especially when the infection occurs in childhood (3). The International Agency for Research on Cancer monograph committee classified *H. pylori* as a Class 1 carcinogen to humans in 1994 (4). Therefore treatment of such an important microorganism is very important. There are many treatment modalities, such as dual, triple

or sequential therapies with some antibiotics, including amoxicillin, clarithromycin, metronidazole, rifabutin and fluoroquinolones, with or without bismuth, using 7 day or 14 day treatment regimens (5,6). The expected or ideal eradication rate is suggested to be 90%, but the actual eradication rates achieved through such treatment are generally much lower. The development of macrolide resistance in *H. pylori* is suggested to be the major cause of treatment failure (7). Furthermore, eradication rates in children are often unsatisfactory and there is a high increase in resistance rates in strains isolated from children (8). The prevalence of resistance of *H. pylori* to clarithromycin among adults is reported 9.3% in Ireland; 19.2% in treatment naïve population and 26% overall in

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France; 10.6% in Taiwan; and 26% among children (9). So the wisest course of action seems to be to check the resistance of the infective organism to a particular drug (metronidazole, clarithromycin), and then commence treatment using the one to which it is most susceptible. Therefore, where available, antimicrobial susceptibility testing should be mandatory before starting eradication therapy, particularly in children. There are various methods to test clarithromycin resistance, such as disc diffusion, agar dilution, E-test, fluorescent in situ hybridization (FISH), restriction fragment length polymorphism (RFLP) and real time polymerase chain reaction (RT-PCR) with melting curve analysis (10). The "gold standard" for accurate diagnosis of *H. pylori* infections is suggested to be either culturing of the pathogen and/or concordant positive results obtained by histology and the rapid urease test, or the ^{13}C -ure breath test (UBT) (6,7,11,12). Since none of these diagnostic tests, except for bacterial culturing, are 100% specific (6), the aim of the study was to compare the efficacy of agar dilution (AD) and two molecular tests FISH and RFLP when testing clarithromycin resistance.

MATERIALS AND METHODS

This study is approved by the Afyon Kocatepe University Medical Faculty Ethical Committee.

Gastric biopsy specimens

Antral biopsy specimens obtained during upper gastrointestinal system endoscopy from adult and pediatric patients were studied. Gastric antrum biopsies were examined for the presence of *H. pylori* and clarithromycin resistance by culture AD, PCR, RFLP and FISH methods. *H. pylori* negative samples according to histopathological examination and patients who were formerly treated for *H. pylori* were excluded.

Culture of *H. pylori*

During the endoscopic procedure, two antral mucosal biopsy specimens were obtained by the use of sterile biopsy forceps. Biopsy specimens were immediately transported to the microbiology laboratory at room temperature. One specimen was cut into small pieces and homogenized in a sterile petri dish. Biopsy particles were smeared on the surface of Columbia agar plates supplemented with 5% sheep's blood. Approximately one hour after endoscopy, biopsy specimens were processed for culturing *H. pylori* in a microaerophilic environment and incubated for 5 to 10 days. *H. pylori* isolates were identified on the basis of characteristic colony morphology, typical appearance on Gram stains, and positive urease, oxidase, and catalase tests. For clarithromycin (Cla) susceptibility testing of *H. pylori* the AD method was used. *H. pylori* strains were suspended in saline and detected with a spectrophotometer. The bacterial suspensions (10^8 colonies per milliliter) were then plated onto agar plates containing various concentrations of clarithromycin. Three days after microaerophilic incubation, minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented visible growth of the bacteria. Clarithromycin >2 µg/mL was determined as the resistance breakpoint.

FISH test

The same formalin-fixed paraffin-embedded gastric biopsies used for histopathological assessment of *H. pylori* were evaluated by FISH. Biopsies were sectioned and dehydrated. Sections were then air-dried and hybridized using the commercially available test system SeaFast Hp Combi Kit (Izinta, Hungary) according to the manufacturer's instructions. Fluorescent-labeled oligonucleotide probes binding to *Hp* 23S rRNA sequences were used for FISH analysis. This test kit labels *H. pylori* with the green fluorescent stain, fluorescein, and resistant *H. pylori* additionally with Cy3-red. The test for clarithromycin resistance is based on three defined point mutations in the 23S rRNA, which can be targeted specifically with the ClaWT, ClaR1, ClaR2, and ClaR3 probes. Different mutations correlate with different MICs of the antibiotics, ClaR1 > 64 mg/L, ClaR2, and ClaR3 between 8 and 64 mg/L. Slides were examined with a BX-60 microscope (Olympus, Germany) equipped with a standard fluorescence filter set. Clarithromycin resistant *H. pylori* was detected with the aid of the Cy3 specific filter (Figure 1-3).

RFLP test

Genomic DNA was extracted from one biopsy sample with QIAamp DNA Micro Kit (Qiagen, Germany). Two pairs of PCR

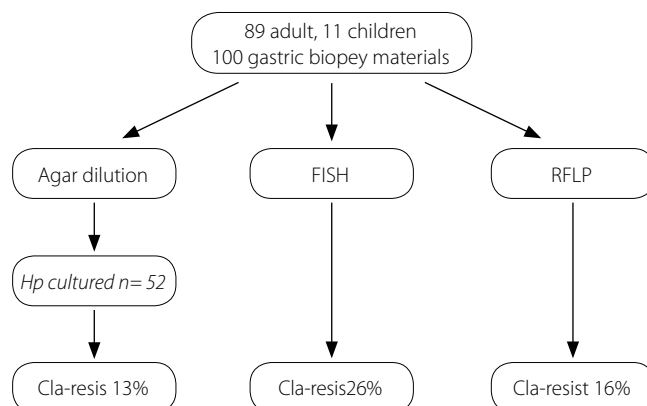


Figure 1. Results of the study

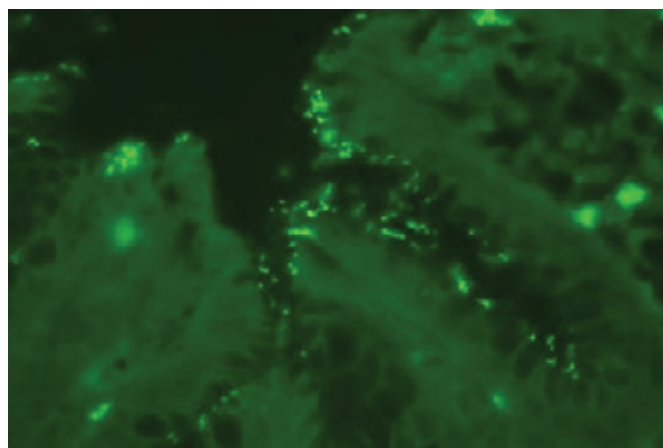


Figure 2. *H. pylori* shown by FISH test (Green filter Fluorescein Dye Absorption 494 nm, Emission 518 nm, target area 16S rRNA) (Red Filter=N2.1 Filter, Cy3 Dye, Absorption 552 nm, Emission 570 nm, target area 23S rRNA)

primers were used to amplify two fragments of the peptidyl-transferase region of the 23S rRNA. The sequences of the primers were based on the published sequence of the 23S rRNA gene of *H. pylori* (GenBank accession number U27270). The primers used for PCR of the 23S rRNA gene of *H. pylori* were F (5'-ATGAATGGCGTAACGAGAT-3'; nucleotides 2051 to 2070) and R (5'-ACACTCAACTTGCG ATTCC-3'; nucleotides 2410 to 2391). PCR was performed in a thermal with a 50-ml volume containing 2 ml of sample DNA, 5 ml of PCR buffer, 0.5 ml each of primer at 25 mM, 0.4 mL of a 25 mM DNTP mixture, 15mM MgCl₂, and 1 U of Taq DNA polymerase. After heating of the mixture at 94°C for 5 min, PCR was performed for 34 cycles, with denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s, followed by final extension incubation at 72°C for 4 min. The mutations responsible for clarithromycin resistance were detected for RFLP with Bsa1 (A2143G) and Bbs1 (A2142G) (New England Biolabs, Inc., Beverly, Mass., U.S.A.) restriction endonucleases. The presence of the mutant was detected by examination of the patterns of the restricted fragments. While PCR products derived from the mutant were digested with either Bsa1 or Bbs1, this was not possible for the wild-type strain. The amplified PCR products were evaluated by gel electrophoresis and photographed on a 1% agarose gel and ethidium bromide fluorescence visualization.

Analysis of test: Sensitivity and specificity of the tests were calculated with the formulas:

$a/(a+c) \times 100$ and $d/(b+d) \times 100$ respectively where a =true positive, b =false positive, c =false negative, d =true negative (13).

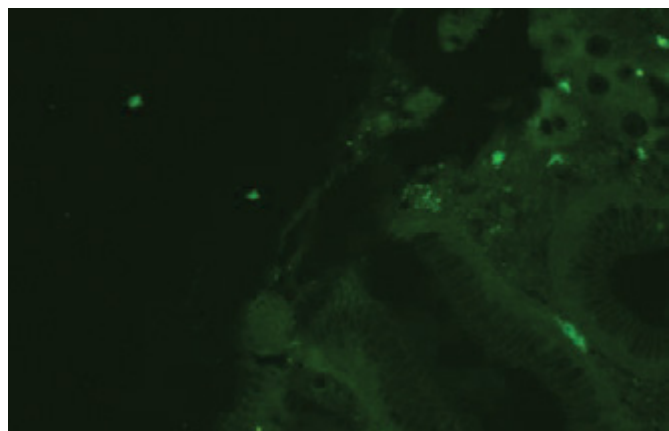


Figure 3. *H. pylori* Negative sample (Green filter Fluorescein Dye Absorption 494 nm, Emission 518 nm, target area 16S rRNA) (Red Filter=N2.1 Filter, Cy3 Dye, Absorption 552 nm, Emission 570 nm, target area 23S rRNA)

Table 1. *H. pylori* clarithromycin resistance in adults and children

	FISH				RFLP		Culture-agar dilution							
	R		S		R		S		R		S		Not cultured	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Adult	23	26	66	74	14	16	75	84	6	7	39	44	44	49
Child	3	27	8	73	2	18	9	82	1	9	6	55	4	36

R: resistant; S: sensitive

RESULTS

A total of 100 patients; 89 adults and 11 children were included in the study. Mean age of the group was 46.2±12.4 (8-68 yrs). Most of the patients had dyspeptic complaints such as abdominal pain, nausea and/or vomiting. On endoscopic examination no peptic ulcer was seen.

Culture on agar plates performed in 100 patients was positive in 52 samples (45 adults, 7 children). Among those 52 samples clarithromycin resistant strains were found in 7 samples; 6 adults, 1 child (13.46%).

The FISH test showed clarithromycin resistance in 26 of 100 biopsy specimens from the antrum, 23 adults, 3 children. The RFLP test showed clarithromycin resistance in 16 of 100 biopsy specimens from the antrum, 14 adults, 2 children. Clarithromycin resistance results detected by 3 tests among adults and children are shown on Table 1.

When the results of the biopsies that were cultured were taken into account, the FISH test had higher sensitivity when compared to AD and RFLP. Both FISH and RFLP had higher diagnostic value. But at the same time FISH had the higher negative predictive value among the 3 tests (Table 2).

The same calculation method was performed by accepting the non cultured results as negative. Then the FISH test had the higher sensitivity and negative predictive value. AD and RFLP had the higher specificity. FISH and RFLP had the higher diagnostic value (Table 3).

DISCUSSION

H. pylori is a common infectious agent particularly in developing countries. It is suggested that the treatment of this microorganism heals gastritis, peptic ulcer disease, and even intestinal metaplasia in some cases (6,14-16). Thus detection and treatment of *H. pylori* is of utmost importance. But the problem is that the microorganism can be resistant to the antibiotics in the eradication therapy at the first administration (primary resistance) or later (secondary resistance). This is important among children, who will receive the eradication therapy for the first time. So when the gastric biopsy is obtained from the *H. pylori* positive patient, antimicrobial susceptibility testing seems essential prior to the first therapy and after the first treatment failure. Culturing, FISH and recently PCR-RFLP are the

Table 2. Calculations made on the basis that only cultured materials are taken into account.

Reference test	Test	sensitivity	specificity	ppv	npv	Diagnostic value
Culture	RFLP	0.8571	0.8889	0.5455	0.9756	0.8846
	FISH	1.0000	0.8444	0.5000	1.0000	0.8654
RFLP	Culture	0.5455	0.9756	0.8571	0.8889	0.8846
	FISH	1.0000	0.8810	0.6154	1.0000	0.9000
FISH	Culture	0.5000	1.0000	1.0000	0.8444	0.8654
	RFLP	0.6154	1.0000	1.0000	0.8810	0.9000

PPV: positive predictive value; NPV: negative predictive value

Table 3. Calculations made on the basis that non cultured materials are accepted as negative

Reference test	test	sensitivity	specificity	ppv	npv	Diagnostic value
Culture	RFLP	0,8571	0,8925	0,3750	0,9881	0,8900
	FISH	1,0000	0,7957	0,2692	1,0000	0,8100
RFLP	Culture	0,3750	0,9881	0,8571	0,8925	0,8900
	FISH	1,0000	0,8810	0,6154	1,0000	0,9000
FISH	Culture	0,2692	1,0000	1,0000	0,7957	0,8100
	RFLP	0,6154	1,0000	1,0000	0,8810	0,9000

PPV: positive predictive value; NPV: negative predictive value

most commonly used and reported methods used to achieve this.

The agar dilution method was suggested as the reference method for susceptibility testing of *H. pylori* (17). In a study from Turkey, cultures grew *H. pylori* in 56 of 102 cases (54.9% sensitivity), and Cla resistance was found in 6 out of 33 *H. pylori* positive biopsies (18.2%) (18). Culture positivity for *H. pylori* is reported between 48.9% and 68% (19,20). In our study, culture positivity was 52%.

There are reports that FISH may be successfully applied for the simultaneous detection of *H. pylori* and macrolide resistance. Rapid and specific detection of *H. pylori* macrolide resistance in gastric tissue by FISH has been reported (19). In our study two molecular methods and the AD method were compared. We found that FISH test indicated a higher Cla resistance of *H. pylori*. PCR-RFLP lay some distance behind in second place whilst agar dilution produced much lower results. There are some studies that compare other methods for determining clarithromycin resistance. Agudo et al. (20) have suggested that RFLP is a fast, accurate and cost-effective test for detection of susceptibility of the strain to clarithromycin. Yet there are few studies about Cla resistance and RFLP. Rüssmann et al. (21) compared FISH test with E-test and disk diffusion. They found the sensitivities of culturing and FISH to be 82.7% and 94.4% respectively. The authors concluded that FISH was a rapid, accurate and cost-effective test for detecting clarithromycin resistance. The authors report that by using FISH they had the results in 3 hours. In a study from Iran, FISH and histological evaluation

were compared for the diagnosis of *H. pylori* infection (13). The authors report the sensitivity and specificity of FISH as 97.9% and 100% respectively.

Caristo et al. (22) reported that clarithromycin resistant strains were found in 66 out of a total of 157 biopsies (42%). They reported that one-third of children were infected with resistant and sensitive strains, and that the resistant strains were in the fundus only. The authors concluded that FISH has some advantages such as that culture preparation is not required, results become available in a short time, and it can be used to test either fresh material or paraffinized old biopsy specimens. On the other hand testing for susceptibility to other antibiotics, which may be performed using cultures, is not possible with FISH.

Clarithromycin resistance is reported as 48.2% by RT-PCR (23), 40.5% by PCR-RFLP (24), 41.9 % by FISH (25), and 23.9% by E-test (26) in Turkey among adults in separate studies. In a very recent study from China primary resistance to clarithromycin was found to be as high as 89% using the PCR method (27).

In a study from Turkey FISH and E-test was compared. Among 31 adult patients' gastric isolates both tests showed resistance in 13 patients (41.9%) (28).

The major advantage of FISH is reported to be the fact that the rRNA-targeted fluorescence-labeled oligonucleotide probes can be used for accurate determination of macrolide susceptibility. This provides the clinician with important information to make a proper treatment recommendation (29-31). It is shown

that E-test is a phenotypic clarithromycin resistance measurement, and FISH test is an established genotypic technique for the detection of *H. pylori* and discrimination between the clarithromycin-susceptible wild type and clarithromycin-resistant mutants (29). FISH test is a reliable fast method for the detection of clarithromycin-resistant *H. pylori* mutants, and results are available within a few hours after an endoscopy.

In conclusion, culturing *H. pylori* is a method requiring a painstaking approach, and following gastric biopsy the material obtained must be sent to the laboratory quickly and without delay under controlled environmental conditions, and the results are only received after a delay of several days. FISH and RFLP tests can be performed from fresh or frozen materials and the results are obtained very quickly, in as little as 3 hours. The probes used for FISH test are commercially available, and the method is cost-effective and can be applied in any laboratory without the need for special equipment or facilities, except for a fluorescence microscope (29-32). So we suggest that in suitable institutions clarithromycin resistance testing should be performed using the FISH test.

Conflict of Interest: No conflict of interest was declared by the authors.

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