The Journal of Pathology: Clinical Research
J Path: Clin Res January 2017; **3:** 29–37
Published online 18 August 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/cjp2.57

Original Article

Rapid identification of *Helicobacter pylori* and assessment of clarithromycin susceptibility from clinical specimens using FISH

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Abstract

Helicobacter pylori **remains one of the most common bacterial infections worldwide. Clarithromycin resistance is the most important cause of *H. pylori* eradication failures. Effective antibiotic therapies in *H. pylori* infection must be rapidly adapted to local resistance patterns. We investigated the prevalence of clarithromycin resistance due to mutations in positions 2142 and 2143 of 23SrRNA gene of *H. pylori* by fluorescence *in situ* hybridisation (FISH), and compared with culture and antimicrobial susceptibility testing in 234 adult patients with dyspepsia who were enrolled. Antrum and corpus biopsy specimens were obtained for rapid urease test, histopathology and culture. Epsilometer test was used to assess clarithromycin susceptibility. *H. pylori* presence and clarithromycin susceptibility were determined by FISH in paraffin-embedded biopsy specimens. We found that 164 (70.1%) patients were positive for *H. pylori* based on clinical criteria, 114 (69.5% CI 62.5–76.6%) were culture positive, and 137 (83.5% CI 77.8–89.2%) were FISH positive. Thus the sensitivity of FISH was significantly superior to that of culture. However specificity was not significantly different (91.4 versus 100.0%, respectively). The resistance rate to clarithromycin for both antrum and corpus was detected in *H. pylori*-positive patients; 20.2% by FISH and 28.0% by E-test.The concordance between E-test and FISH was only 89.5% due to the presence of point mutations different from A2143G, A2142G or A2142C. We conclude that FISH is significantly more sensitive than culture and the E-test for the detection of *H. pylori* and for rapid determinination of claritromycin susceptibility. The superior hybridisation efficiency of FISH is becoming an emerging molecular tool as a reliable, rapid and sensitive method for the detection and visualisation of *H. pylori*, especially when the management of *H. pylori* eradication therapy is necessary. This is particularly important for the treatment of patients with *H. pylori* eradication failure.**

Keywords: clarithromycin resistance; FISH; *H. pylori* detection

Received 17 June 2016; Accepted 12 August 2016

The authors report no conflicts of interest. The authors are all responsible for the content and writing of the paper.

Introduction

Helicobacter pylori is the aetiological agent of gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [1–8], and its eradication depends on the choice of antibiotics to which the organism is susceptible [1,4,9]. Disease outcome in *H. pylori* is associated with several virulence factors [10] including CagA, which is present in 60% of the strains [11]. Triple therapy includes a proton pump inhibitor (PPI) in combination with two antibiotics: amoxicillin, clarithromycin or metranidazole [8,12–19]. Some authors recommend the use of metranidazole instead of clarithromycin in regions where the resistance to this antibiotic exceeds 15–20% [13,16,19]. The recent Maastricht IV Consensus

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reports and other studies recommended more than seven days of triple therapy for eradication of *H. pylori*. Treatment of *H. pylori* is important since gastric cancer risk decreases significantly in patients without pre-malignant lesions who receive treatment [1], and also has a low relapse rate in patients with duodenal ulcer [20]. Because *H. pylori* eradication failure is mainly associated with clarithromycin resistance, it is important to know the prevalence of resistance to this antibiotic in the different regions of the world [7,21,22]. The prevalence of clarithromycin resistant *H. pylori* is as high as 10% in France and Belgium, 27% in Italy [23] and 24.2% in Turkey [24].

Clarithromycin binds to the 50S ribosomal subunit in the 23S rRNA and inhibits protein synthesis [4,18,23,25–28]. Resistance to clarithromycin is associated with three main point mutations at positions A to G at 2142, 2143 and A to C at 2142 of the 23S rRNA gene [4,6,17,23,25,29–34]. All antibiotic resistance mechanisms in *H. pylori* seem to be chromosomally mediated [27]. Novel technologies that include *in situ* hybridisation for clarithromycin resistance on gastric biopsies are excellent options if culture is not possible [35–37].

Traditional culture methodology is expensive and rarely available, therefore, antibiotic susceptibility testing is not performed routinely. Agar dilution, broth dilution, disc diffusion test and Epsilometer test (E-test) are phenotypic methods used for assessment of clarithromycin susceptibility, but there is a need to obtain fast and more sensitive results using molecular tests rather than phenotypic methods [35,36]. Fluorescence *in situ* hybridisation (FISH) is a molecular technique that combines the detection of *H. pylori* and the determination of clarithromycin susceptibility, and correlates well with the results obtained by traditional culture methodology and clarithromycin susceptibility assay by E-test as recommended in the Maastricht IV Consensus Report [2,4,19,38].

FISH allows the morphology of whole bacteria to be seen [5]. FISH can be performed on formalin-fixed paraffin embedded tissue, on frozen antrum and corpus gastric biopsies, or isolated *H. pylori* colonies using fluorescence-labeled oligonucleotide probes which hybridise to specific rRNA sequences [2,5,6,8,15,22,39]. A major limitation is that the molecular basis for clarithromycin resistance may differ by country so the system needs to be individualised and occasionally checked against culture [36]. However, more than 90% of the clarithromycin resistant *H. pylori* isolates have been associated with the three common point mutations mentioned above and which are included in FISH [40]. In this study, we evaluated the efficacy of FISH for the detection of *H. pylori*, and for the determination of clarithromycin resistance due to mutations in the 2142 and 2143 positions of 23S rRNA gene. The results were compared with traditional culture and antimicrobial susceptibility testing results.

Methods

Patients

Two hundred and thirty four patients with dyspepsia (65 male, 169 female; mean age 43.8 ± 14.0 years, age range 17–83) were admitted to the Outpatient Gastroenterology Clinic and Endoscopy Unit at Dokuz Eylül University Hospital and referred to upper endoscopy between April 2006 and February 2011. The patients included in the study were treatment naïve before endoscopy was performed. Patients were excluded if they had received antibiotics in the previous month, had received proton pump inhibitors (PPIs), or had prior gastric surgery. Patients were also excluded if they were pregnant or had gastrointestinal malignancy, alcohol abuse, drug addiction, or chronic use of corticosteroids or nonsteroidal anti-inflammatory drugs. All patients provided written informed consent to participate in this study and demographic information was obtained.

Endoscopy and gastric biopsy sampling

Three antrum and corpus biopsies were taken from each patient: one set of antrum and corpus biopsies was used for rapid urease test (RUT); a second set was fixed and transported in 10% formalin solution for histopathological examination. Finally the last set of biopsies was immediately transported to the Medical Microbiology Laboratory and processed for culture.

Rapid urease test

One biopsy from the antrum and one biopsy from the corpus were used for RUT. Antrum and corpus biopsy specimens were placed separately in tubes containing urea solution, and then two drops of 1% (vol/vol) phenol red solution were added. If the indicator solution changed from yellow to pink, it was considered a positive result. If the indicator did not change, it was considered a negative result. The results of this house-made RUT were recorded in less than 24 h.

Histopathological examination of biopsy specimens

Paraffin-embedded gastric biopsy specimens were routinely processed. Haematoxylin and eosin, Alcian

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blue and Giemsa stains were used for grading bacterial density and gastritis activity according to the updated Sydney System [41]. A four-point grade scale of none (grade 0), mild (grade 1), moderate (grade 2) and severe (grade 3) was used to score the presence of chronic superficial gastritis, active gastritis, gastric atrophy, intestinal metaplasia (IM) and *Helicobacter*-like organisms (HLO). Histopathology evaluation for all patients was performed by a single pathologist (SS) for consistency and she was unaware of the results of culture and FISH.

H. pylori status criteria used

We used the test-and-treat criteria, which dictate that any positive test for *H. pylori* should be interpreted as patient positive for *H. pylori* infection [19].

H. pylori status was defined as positive when one of two diagnostic tests – RUT and/or histopathology – were positive. A patient was classified as being *H. pylori* negative when histopathological examination and RUT were both negative.

H. pylori culture

Antrum and corpus biopsies were cultured either onto (1) Tripticase soy agar containing 5% (vol/vol) sheep blood (TSA) (Beckton Dickinson, [BD] Sparks MD, USA), and Skirrow Medium (BD) containing antibiotics (Trimethoprim, Vancomycin, and Polymixin B) supplemented with 5% (vol/vol) horse blood (BD), or (2) Columbia Blood Agar (Oxoid, Basingstoke, Hampshire, England) containing 7% (vol/vol) defibrinated horse blood (Oxoid) and *H. pylori* selective supplement (DENT) (Oxoid). Plates were incubated at 37 °C in an anaerobic jar containing GasPak Campy Container System (BD) for 3–7 days under microaerophilic conditions. In the case of no growth at the seventh day, all cultures were held for up to 14 days which is required for initial isolation. After incubation, colony morphology, microscopic examination for motility, and Gram staining for morphology were documented and urease, catalase, and oxidase tests were performed to identify *H. pylori*. *H. pylori* strains were stored at −80 °C in brain heart infusion (BHI) broth (Oxoid) containing 20% (vol/vol) glycerol.

H. pylori NCTC 11637 reference strain was used in this study as a positive control for culture and antimicrobial susceptibility testing in the E-test.

Antimicrobial susceptibility testing

Gradient diffusion test (E-test) (AB Biodisk, Solna, Sweden) was used to assess clarithromycin susceptibility in isolated *H. pylori* strains. The bacterial inoculum was prepared in Brucella Broth (BBL) from subcultures grown on Columbia Blood Agar (Oxoid) containing 7% (vol/vol) defibrinated horse blood (Oxoid) and *H. pylori* selective supplement (DENT) (Oxoid). The Mueller-Hinton agar (Oxoid) supplemented with 5% (vol/vol) defibrinated sheep blood (Oxoid) was used to inoculate 100 µl of *H. pylori* culture suspension of a McFarland standard 3 ($\sim 10^9$ CFU/ml) turbidity in Brucella broth (BBL). Plates with clarithromycin E-test strips were incubated at 37 °C in a jar including the GasPak Campy Container System (BD) under microaerophilic conditions for 72 h. The minimum inhibitory concentration (MIC) was considered the lowest concentration of the drug that inhibited visible growth of *H. pylori*. Isolates were defined as clarithromycin resistant when the MIC was ≥ 1 µg/ml, susceptible when MIC was <0.5 µg/ml, and intermediate when MIC was 0.5–1 µg/ml according to Clinical Laboratory Standard Institutes (CLSI) recommendation [42]. However, these breakpoints were defined only for the agar dilution method and they have not yet been established for the E-test. A plate without antibiotic strip was used to confirm the purity of the culture and the lack of contamination.

FISH

Paraffin-embedded antrum and corpus biopsy sections were examined by FISH (BactFISH *H. pylori* Combi-Kit IZINTA Trading Co. Ltd., Budapest, Hungary) as previously described [22]. This kit detects several point mutations of the 23S rRNA gene of *H. pylori* at positions A2143G, A2144G, A2143C. In brief, formalin-fixed, paraffin-embedded biopsy sections were deparaffinised [6,15,17,22,39]. After air-drying, 40 μl of DNA hybridisation solution containing FISH probes 5′ labeled with FITC or Cy3 was added at 46 °C for 90 min for hybridisation. The hybridised slide was rinsed with wash buffer to prevent non-specific hybridisation and washed for 15 min at 48 °C in a high-density polyethylene coplin staining jar with plastic screw cap (SIGMA Co. Saint Louis MO, USA). Air-dried slides were counterstained with 10 µl of 4′, 6′-diamidino-2-phenylindole (DAPI/Antifade-MC QBIOgene) to visualise DNA for 5 min at room temperature [7,22]. Probe specificity was confirmed using control slides as previously reported [22]. *H. pylori* was visualised with a Nikon Eclipse E600 epifluorescence microscope (Nikon Corp., Tokyo, Japan) that included five different filter sets for DAPI, FITC, rhodamine, dual band (FITC/Rhodamine), triple band (DAPI/FITC/

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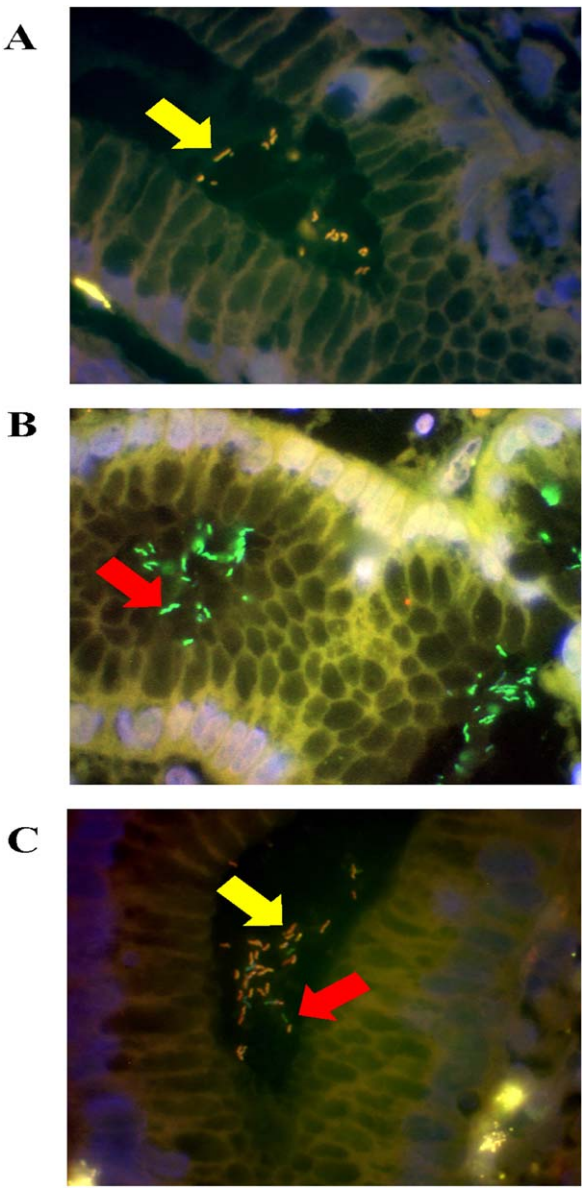


Figure 1. Detection of *H. pylori* and determination of clarithromycin susceptibility in gastric biopsies from three different patients by FISH. Probes were visualised using a triple filter. Green fluorescence indicates clarithromycin sensitive *H. pylori* (B and C); yellow fluorescence indicates clarithromycin resistant *H. pylori* (A and C). The DAPI counterstain produces blue fluorescence (A, B and C). Mixed infection is present within the same biopsy specimen in panel C. Arrows indicate the presence of *H. pylori* infecting the gastric mucosa (Magnification, X100).

Rhodamine) automatised inter-changeable filters. Individual single-colour images were captured through a high-sensitivity monochrome charge-coupled device (CCD) camera (Figure 1). Quantification of *H. pylori* was performed using a semiquantitative grading system (1+ to 3+). Digitally captured individual image photographs were overlaid and processed with MacProbe imaging software (PSI Scientific Systems, USA).

Extraction of *H. pylori* genomic DNA

Bacterial genomic DNA was obtained from isolated *H. pylori* strains. DNA was extracted using the Wizard Genomic DNA isolation kit (Promega Madison, WI, USA), or the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). All DNA products were measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific Pittsburgh, PA, USA).

Determination of *H. pylori* and cagA positivity by PCR

H. pylori strains were determined for the presence of the *ureA* gene by HPU PCR with the primers HPU-1 (5′-GCCAATGGTAAATTAGTT-3′) and HPU-2 (5′-CTCCTTAATTGTTTTTAC-3′) [33]. The presence of the *cagA* gene was determined by CagA PCR with the primers cagA2530S (5′-GTTAARAATRGTGTRAAYGG-3′) and cagA3000AS (5′-TTTAGCTTCTGATACCGC-3′) [43]. PCR conditions have been previously reported [43,44].

Statistical analysis

The McNemar's χ^2 test was used. The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) were calculated (SPSS version 15.0). The results were considered to be significant at a p-value of <0.05. Fisher exact test and Kappa confidence were also used.

Ethics

The research protocol for this study was approved by the Institutional Review Board, and the Ethical Committee, of the Dokuz Eylül University, Faculty of Medicine (13.07.2006/166).

Results

One hundred sixty four of 234 patients (70.1%) were diagnosed as *H. pylori* positive by histopathology and/or RUT methods. The descriptive demographic information of *H. pylori* positive and *H. pylori* negative patients is shown in Table 1. Amongst 164 *H. pylori* positive patients, 93.9% were positive by both histology and RUT (Table 2). By culture, 114 (69.5%) patients were positive. The sensitivity,

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Table 1. Demographic characteristics of 234 dyspeptic Turkish patients according to *H. pylori* status

	H. pylori infection ($n = 234$)	
	Negative	Positive
Number	70	164
Mean age	43.1 + 15.6	44.0 + 13.3
Median age	43.5	46
Age range	20–83	17–75
F:M ratio	2.89	2.49

specificity, positive predictive value (PPV) and negative predictive value (NPV) for culture were 69.5, 100.0, 100.0, and 58.3%, respectively ($\kappa = 0.577$). We compared the two different culture methods for *H. pylori*. For the first part of the study 64 patients or 128 biopsies of antrum and corpus were processed in NYU School of Medicine and the last 340 gastric biopsies of 170 patients were processed at Izmir, Dokuz Eylül University Faculty of Medicine. We found no significant differences in the recovery of *H. pylori* by location (56.3% in New York versus 53.5% in Izmir).

Of 164 *H. pylori* positive patients, 137 (83.5%) were *H. pylori* positive by FISH method. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of FISH were 83.5, 91.4, 95.8 and 70.3%, respectively ($\kappa = 0.690$). Despite the high specificity and positive predictive value of culture, the FISH method was able to detect significantly more *H. pylori* positive subjects than culture (83.5 versus 69.5%, $p = 0.004$). The sensitivity of FISH and the patchy distribution of *H. pylori* infection allowed the identification of two positive FISH subjects who were negative by traditional histology (Table 2).

Susceptibility testing was only possible in 114 (69.5%) patients with positive cultures. In contrast, we evaluated 137 (83.5%) patients by FISH due to the higher sensitivity of this method. We assessed clarithromycin susceptibility by E-test in the 114 culture positive patients, and found 32 patients (28.0%) carrying *H. pylori* strains resistant to clarithromycin. On the other hand, when we assessed *H. pylori* clarithromycin susceptibility by FISH in the same patients, resistance was demonstrated in 23 (20.2%) of those patients. The difference in patients with resistant strains between the two methods was not significant. The concordance between E-test and FISH was 89.5% (Table 3).

Table 2. Biopsy-based results of the 164 patients identified as *H. pylori* positive

Histology	RUT	Number (%)	FISH (%)	Culture (%)
Negative	Positive	4 (2.4)	2 (50.0)	4 (100.0)
Positive	Negative	7 (4.3)	6 (85.7)	6 (85.7)
Positive	Positive	153 (93.3)	129 (84.3)	104 (68.0)

RUT = rapid urease test; FISH = fluorescence *in situ* hybridisation.

Next, we compared the prevalence of clarithromycin resistant strains by specific location in the stomach. *H. pylori* isolates were recovered from 102 antrum biopsies (89.5%) and from 96 corpus biopsies (84.2%) in the 114 culture positive patients. Amongst antrum positive *H. pylori* isolates, we identified 26 (25.5%) clarithromycin-resistant strains by E-test but only 15 (14.7%) by FISH. This result suggests that FISH has lower sensitivity for the detection of clarithromycin resistant *H. pylori* strains in antrum biopsies although the difference was not significant (p-value = 0.06). Amongst the corpus biopsy results, we identified 20 (20.8%) clarithromycin-resistant *H. pylori* strains by E-test and 15 (15.6%) by FISH. The differences observed between the methods in the corpus were also not significant (Table 4). An interesting observation was that in two patients with FISH positive results but negative culture results, we observed clarithromycin-resistant coccoid forms. However, similar coccoid forms were observed in four patients positive for both FISH and culture that were either sensitive or resistant to clarithromycin. Therefore, bacterial morphology may not explain why some culture negative patients had positive FISH results. *H. pylori* strains were confirmed by PCR of the *ureA* gene with the primers HPU-1 and HPU-2. Confirmed *H. pylori* strains were assessed for the presence of the *cagA* gene. Amongst 114 *H. pylori*-isolated strains, 53 (46.5%) were *cagA* positive. CagA status was compared with clarithromycin susceptibility in antrum and corpus biopsy sections. There were no differences between *cag*A positivity and clarithromycin susceptibility in antrum and corpus biopsy sections (Fisher exact test, p = NS)

Discussion

The diagnosis of *H. pylori* is based on non-invasive and invasive tests. However, a major limitation of invasive tests is that *H. pylori* infection has a patchy distribution, and the likelihood of recovering or visualising the bacteria increases with each additional biopsy specimen studied from the same patient [35]. When gastric biopsies are evaluated by a specialised pathologist, the sensitivity and specificity of histopathology for the diagnosis of *H. pylori* can be very high [36]. For other tests such as RUT, its sensitivity depends on bacterial load. It has been published that at least 10^5 bacteria are necessary for a positive

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Table 3. Comparison of E-test and FISH results for both antrum and corpus biopsy specimens in 114 culture positive patients

Results obtainedby FISH	Number of strains (%)	Clarithromycin by E-test	
		Resistant (%)	Sensitive (%)
Positive for clarithromycin resistance	23 (20.2)	19 (82.6)	4 (17.4)
Positive for clarithromycin sensitive	87 (76.3)	12 (13.8)	75 (86.2)
Negative for *H. pylori*	4 (3.5)	1 (25.0)	3 (75.0)

FISH = fluorescence *in situ* hybridisation.

RUT. Furthermore, there is a possibility that other urease positive gastric *Helicobacter* and non *Helicobacter* species may be responsible for the positive urease test, but their prevalence is <1% in gastric biopsies [45,46]. The success of *H. pylori* recovery depends on several factors that affect the sensitivity of *H. pylori* isolation, including slow *in vitro* growth of the bacteria, the sensitivity to PPIs and other medications that affect bacteria load [35]. All these factors may explain the small number of specific laboratories that are capable of isolating the organism from clinical gastric biopsies [36]. In the success of *H. pylori* eradication, we need to obtain correct information on the susceptibility of strains to antimicrobials and this represents a big challenge for clinical microbiologists and gastroenterologists.

In many cases it is imperative to culture *H. pylori* and determine its antimicrobial susceptibility to guarantee the success of the therapy.

In this study, we compared FISH and traditional culture methods and, according to our results, FISH was a more sensitive method than culture for the detection of *H. pylori* (83.5 versus 69.5%, respectively, $p = 0.004$). Furthermore, the specificity of FISH was 91.4%. Our specificity result is comparable with the Samarbaf-Zadeh *et al* study in which the specificity of FISH for the detection of *H. pylori* was 100% [5], and with other reports on specificity of FISH for the detection of *H. pylori* that were >92 and 97%, respectively [12,15].

We found 32 patients with negative cultures in whom *H. pylori* was detected by FISH. Failure to culture *H. pylori* may be due to contamination with other bacteria or the patchy distribution of *H. pylori* infection in the stomach [2]. Negative culture due to low bacterial load, contamination, coccoid forms or transport delay is also possible. This is an important problem in the clinical setting [7]. The stability of *H. pylori* in biopsy material during transport is a limiting factor for culture that does not affect FISH results [17], and it is possible to detect *H. pylori* by FISH in culture negative patients. For this reason, we believe that FISH is better than culture methods for the management of treatment therapy. The fact that we found four culture positive but FISH negative patients was an intriguing finding that may be explained by the patchy distribution of *H. pylori* infection or a low level of colonisation in the biopsy used for FISH. Rüssmann *et al* reported that FISH may fail to detect *H. pylori* in biopsies with fewer than 10 colony forming units (CFU) (4.0 ±1.4 CFU) whilst the site-specific culture was positive [17].

The results of clarithromycin susceptibility by FISH were compared with the results of clarithromycin susceptibility by E-test (Table 3). We detected four strains as clarithromycin resistant with FISH but clarithromycin sensitive by E-test. Discrepant results between FISH and E-test can be explained by the fact that the E-test, and other traditional methods, base their results on a single isolated colony whilst FISH tests multiple bacterial strains by *in situ* hybridisation, making it a more nuanced, accurate methodology. We also observed 12 other discrepant results, in which *H. pylori* strains were clarithromycin resistant by E-test but clarithromycin-susceptible by FISH. One explanation of these results is that the FISH method used in this study only detected clarithromycin resistance with point mutations in the 2143 and 2142 positions of 23S rRNA. Therefore, it is possible that the 12 strains resistant to clarithromycin by E-

Table 4. The comparison of E-test and FISH results for specific biopsy specimens in *H. pylori* positive patients

Results by FISH	Antrum biopsy ($n = 102$)			Corpus biopsy ($n = 96$)		
		Clarithromycin by E-test			Clarithromycin by E-test	
	Number of strains (%)	Resistant (%)	Sensitive (%)	Number of strains (%)	Resistant (%)	Sensitive (%)
Positive for clarithromycin resistance	15 (14.7)	13 (86.7)	2 (13.3)	15 (15.6)	12 (80.0)	3 (20.0)
Positive for clarithromycin sensitive	83 (81.4)	11 (13.3)	72 (86.7)	73 (76.1)	7 (9.6)	66 (90.4)
Negative for *H. pylori*	4 (3.9)	2 (50.0)	2 (50.0)	8 (8.3)	1 (12.5)	7 (87.5)

FISH = fluorescence *in situ* hybridisation.

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test contain point mutations different from the mutations in the 2142 and 2143 positions. When we sequenced the 23S rRNA region between 2060 and 2690 bp in the discrepant strains, our results showed that two strains had the same A2143G or A2142G point mutations and should have been identified. For three other strains, we identified point mutations at C2131T, C2310A and C2428T, but none of those point mutations has been implicated in clarithromycin resistance. However, since we only sequenced a partial region of the 23S rRNA gene, our results do not exclude the possibility of point mutations in the rest of the 23S rRNA that was not sequenced. Based on this observation, we need to emphasis to clinicians the limitations of FISH because not all the clarithromycin resistance can be explained by the three point mutations that we investigated here.

Furthermore, clarithromycin-resistant strains without mutation in 23S rRNA may exist, therefore unknown genes outside 23S rRNA could be involved in clarithromycin resistance [47].

Results of clarithromycin susceptibility by biopsy location yielded 26 (25.5%) clarithromycin-resistant *H. pylori* isolates by E-test but only 15 (14.7%) by FISH in antrum biopsies. Amongst corpus biopsies, we found 20 (20.8%) clarithromycin-resistant *H. pylori* strains by E-test and 15 (15.6%) by FISH. Although the differences observed between the methods in the corpus were not significant, we found a significant difference in the sensitivity between E-test and FISH in the antrum. Despite that, no topographic site differences in the stomach have been reported. Several authors have indicated that specific *bab*AB genotype correlates with colonisation of the antrum [48], or specific chemotaxis to the antrum is controlled by some genes (TLpD) [49].

When antrum and corpus results were combined, we detected 10 (8.8%) clarithromycin-susceptible and –resistant strains (mixed infections) by E-test and 14 (12.3%) clarithromycin-susceptible and -resistant strains (mixed infections) by FISH. Therefore, the presence of mixed infection in this study was estimated to be higher than 10% and this phenomenon may help to explain some failures in treatment.

We found that clarithromycin resistance was 28.0% by E-test and 20.2% by FISH. We also found a good correlation for sensitive strains (89.5%) between both methods. Our results are not comparable with the results of Vega *et al* [7], who utilised frozen gastric biopsies to compare E-test with FISH, and found that sensitivity and specificity of FISH was 90 and 100%; respectively. The authors found only two discrepant results between FISH and E-test and not mixed infections. However, they did not perform FISH in culture negative patients [7]. Rüssmann *et al* found no discrepancies between FISH, E-test and disc diffusion [39]. In our study, we observed discrepancies in four patients in whom we isolated clarithromycin-susceptible strains by E-test, but FISH detected the presence of clarithromycin-susceptible and –resistant strains. Therefore, these discrepant results may be explained by the fact that mixed populations were present or sensitive strains were present at low concentrations and could not be detected by the E-test method. In this study, if susceptible and resistant strains were present in the same patient, he or she was considered colonised with a resistant strain. Similar discrepant results were found by Cerqueira et using a novel method of peptide nucleic acid probes to detect clarithromycin resistant *H. pylori* strains when compared with E-test [50].

In 137 *H. pylori* FISH positive patients, we found 111 (81.0%) patients infected with a clarithromycin-susceptible strain, 11 (8.0%) patients infected with a clarithromycin-resistant strain and 15 (11.0%) patients infected with both clarithromycin-susceptible and –resistant strains, as determined by FISH. One possible explanation for the results in the last group is that there are two copies of the 23S rRNA gene in *H. pylori*. Both copies of the mutated 23S rRNA gene frequently exhibit the same mutation, but heterozygotes (one copy of the wild-type and one copy of the mutated gene) have sometimes been observed [18,23]. In addition, Toracchio *et al* reported the colonisation by clarithromycin sensitive and resistant *H. pylori* strains in the same geographic area and in the same patients [28].

Trebesius *et al* found that FISH could be a powerful tool to successfully hybridise 'culture resistant' coccoid forms of *H. pylori* in the stomach [6]. In our study, we found two patients with FISH positive but culture negative results showing clarithromycin-resistant coccoid forms. However, we also found FISH and culture positive patients who showed clarithromycin-sensitive coccoid forms. Thus, morphology (coccoid forms) does not explain FISH positive but culture negative results in those patients.

Finally, the prevalence of *cag*A-positive strains was 46.5%. A previous report from Turkey showed a CagA prevalence of 82% [51], which is almost twice the prevalence found in this study. We do not have an explanation for this difference.

In conclusion, FISH appears to be a good technique that simultaneously determines *H. pylori* status and clarithromycin susceptibility with better sensitivity and specificity than traditional culture methods. FISH can help clinicians to manage the treatment of *H. pylori*

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infected patients. When clarithromycin resistant strains are detected by FISH, clinicians should be able to select the best treatment for *H. pylori* eradication. Because of the alarming increase in clarithromycin and other antibiotic resistance in *H. pylori* strains, antimicrobial susceptibility testing is imperative.

Acknowledgements

This research was supported by Dokuz Eylül University Research Foundation grant 2006.KB.SAG.042 and also supported in part by R01 GM63270 by the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. EDG was the recipient of a PhD fellowship from the Scientific and Technological Research Council of Turkey (TUBITAK). We thank Isabel Teitler and Kartik Raju for their revision of the English style of this manuscript.

Author contributions

EDG, CG, SS, MS, ST, OA, IS and AZO participated actively in the acquisition of data, analysis and interpretation, participated also in revising the document and provided final approval of the version to be published. OY and GIPP participated in the conception and design of the study, drafting the article and provided final approval of the version to be published.

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